Vol. 42, No. 1 January, 1955

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

PUBLISHED BIMONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutlant, Vt.
U.S.A.

Maruzen Co. Ltd.
Tokyo,
Japan

Santo Vanasio
Köln
Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published bimonthly, one volume per year.

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The Journal of Biochemistry, Vol. 42, No. 1, 1955

DETECTION OF XANTHURENIC ACID IN DIAZO URINE OF SEVERE TUBERCULOUS PATIENT

By KATASHI MAKINO and HITOSHI TAKAHASHI

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(Received for publication, October 2, 1954)

It was confirmed by Makino *et al.* (1) that the principal substance in the urine giving Ehrlich's diazo reaction is 3-hydroxykynurenine. They also reported that kynurenine and other tryptophan metabolites were remarkably excreted in the same urine and suggested the existence of xanthurenic acid in the same urine. Recently we could confirm the excretion of xanthurenic acid in the urine of severe tuberculous patient giving Ehrlich's diazo reaction.

EXPERIMENTAL

400 ml. of urine giving a strong diazo reaction and the urochromogen test of Weiss, was saturated with ammonium sulphate and filtered. Then it was shaken vigorously with 120 ml. of 90 per cent phenol. After standing the separated phenol layer was shaken with a mixture of 150 ml. of ether with 40 ml. of water. After standing again the separated water layer was washed with 10 ml. of ether and concentrated in an atmosphere of hydrogen under the reduced pressure to a small volume. This was treated with alcohol for desaltation and again concentrated to a syrup, which was paper chromatographed with the supernatant of the mixture of butanol, acetic acid and water in ratio of 4:1:5. Several fluorescent spots were found besides kynurenine (Rf 0.41) and 3-hydroxykynurenine (Rf 0.37). When this paper chromatogram was sprayed with diazotized sulphanilic acid, three red spots having Rf 0.37 (3-hydroxykynurenine), 0.45 and 0.75 (dimmly) were recognized. The second spot which had the same Rf value as that of the synthetic xanthurenic acid, showing a purple fluorescence, and a bluish green color with ferric chloride solution, was eluted. This eluate on development with 70 per cent iso-propanol (acidified with acetic acid) separated into two spots, one of which showed Rf 0.7 and gave a bluish green color with ferric chloride solution, and the other gave a weak greenish brown color with ferric chloride and had the same Rf (0.64) with an authentic sample of xanthurenic acid. The latter spot was eluted and subjected to the further investigation with paper chromatography and identified with xanthurenic acid as indicated in the following table.

TABLE I

Rf Value of the Second Red Spot

Developer	The second red spot	Xantureni acid
Butanol—acetic acid—water (4 : 1 : 5)	0.45	0.45
70 per cent iso-propanol (acidic)	0.64	0.65
Methanol—butanol—benzene—water (2 : 1 : 1 : 1)	0.63	0.63
Butanol saturated with 1 per cent NH_4OH	0.03	0.03

We thank Prof. T. Sakan for a gift of xanthurenic acid. This work was aided by a grant from the scientific research fund of the ministry of Education of Japan.

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The Journal of Biochemistry, Vol. 42, No. 1, 1955

NEW COLORIMETRIC DETERMINATION OF CALCIUM AND MAGNESIUM

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(Received for publication, October 2, 1954)

For the determination of Ca, the methods of Kramer and Tisdall (*1*), Clark and Collip (*2*), Sobel (*3, 4*), Sendroy (*5, 6*), and Roe and Kahn (*7*), *etc.* are generally used, whereby Ca is precipitated as an oxalate or a phosphate salt, and determined indirectly from the amount of oxalic acid or phosphoric acid. These methods are however, difficult, requiring complicated and time consuming operations. For the determination of both dialytic and non-dialytic Ca in serum, there is no convenient method other than that of Toksoy and Eser (*8*).

Denis (*9*), and the Titan Yellow (*10, 11, 12*) methods have been used for the determination of Mg, but these are not altogether satisfactory, and no outstanding report has yet appeared on the determination of dialytic and nondialytic Mg.

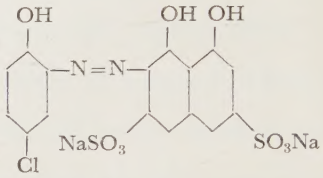
Recently Shwarzenbach (*13*) published the ethylene diamine tetraacetate method for determining the hardness of water (the method has already been adopted in our country.) Even by this method, it is difficult to separate Ca from Mg, and the method is not to be applied microestimation.

After several years of studying the determination of Ca and Mg, the following direct method was devised (*14–21*). Compared with others, it is believed to be simpler, highly sensitive, and of wide application, covering not only the determination of Ca and Mg in serum (*14–18*), food (*19*), mineral water (*20*), hardness of water (*20, 21*), but also the determination of dialytic Ca (Ca ion) (*16, 24*), and dialytic Mg (Mg ion) (*18*) in serum. The determination of the latter is hardly feasible with other methods, not even with the more complicated ones.

The principle of the proposed method (Yanagisawa's method) is explained in what follows.

The colouring reagent used is an alkaline solution of disodium 1-hydroxy-4-chloro-2,2-diazobenzene-1,8-hydroxy-naphtalene-3,6 disulfonic acid, its natural color is purple ; and it becomes red in the presence

of Ca or Mg. In strong alkaline solution, Mg forms $Mg(OH)_2$, thereby releasing the reagent particles which then attach themselves only to Ca ions present in the solution. This phenomenon constitutes the basis of the proposed method.



I. Determination of Ca in Serum

Reagents—

A) 0.00025 *M* disodium 1-hydroxy-4-chloro-2, 2-diazobenzene-1, 8-dihydroxy-naphtalene-3, 6-disulfonic acid. Weigh exactly 0.1298 g. of the pure reagent above; add 10 ml. of 0.01 per cent hydrochloric acid solution in a 100 ml. volumetric flask. Dilute it exactly to 100 ml. with distilled water (refer to this as stock solution); dilute 10 ml. of this stock solution to 100 ml. with distilled water; store the solution in a brown bottle and keep in a dark place. This solution will keep for a month.

B) Sodium hydroxide, volumetric 2 *N*.

C) Standard solution:

Weigh exactly 0.2497 g. of pure calcium carbonate $CaCO_3$; dissolve this into a minimal quantity of diluted hydrochloric acid; dilute the full amount of solution to one liter with distilled water. One ml. of this solution contains 100 γ of Ca. Dilute with four parts distilled water; then 1 ml. of the final dilution contains 20 γ of Ca.

Standard Curve—Place 0, 0.1, 0.2, 0.3, 0.4, and 0.5 ml. of Ca standard solution into 6 test tubes, and dilute each test tube to 0.5 ml. with distilled water. These tubes then contain 0, 2, 4, 6, 8, and 10 γ of Ca, respectively. Add 2.0 ml. of regent (A) and 2.5 ml. of reagent (B) into the content of each test tube and transfer the mixture to cuvette. Use Coleman Spectrophotometer. The zero concentration of Ca is used as the blank solution. Adjust the reading for the blank solution to 30 per cent transmission with a 620 mμ-filter. Take readings for each cuvette. Plot these readings on the log scale of a semi-log paper; these should fall on a straight line up to 12 γ of Ca. Though some difference may exist among types of electrophotometers, more accuracy may be attained by shifting the transmission point for the blank solution to 40 per cent or sometimes to 50 per cent.

Procedure—Take 0.4 ml. of distilled water into a clean test tube. Take exactly 0.1 ml. of fresh serum with a pipette and add to the above distilled water. Thoroughly clean inside of the pipette. Place 0.5 ml. of distilled water into another test tube and use this for comparing blank solution. Add 2 ml. of reagent (A) and 2.5 ml. of reagent (B) into each test tube. Read off the photometric scale applying a 620 mμ-filter and fixing the calibration for the blank solution at 30 per cent transmission point as formerly.

Calculation—The amount of Ca in the serum is calculated from the standard curve drawn in Fig. 1 corresponding to the given photometric reading. (Since 10γ of Ca should be contained in 0.1 ml. of normal serum, approximately 10 mg. of Ca should be contained in 100 ml. of serum; the multiplying factor of x 1000 should be kept in mind.) In practice, some shift of the standard curve may occur because of errors of pipette or discolouring of the dye, so that a check or recalibration of the standard curve is recommended: measure and check 10 γ of Ca each time and compare.*

II. Determination of Dialytic Ca in Serum

Reagents—

A) 0.00025 *M.* disodium 1-hydroxy-4-chloro-2, 2-diazobenzene-1, 8-dihydroxy-naphtalene-3, 6-disulfonic acid. The same reagent as for the determination of Ca in serum.

B) Sodium hydroxide, volumetric 2 *N.*

C) Ammonium oxalate, 4 per cent aqueous.

Dilute 4 g. of pure ammonium oxalate to 100 ml. with distilled water.—Caution should be exercised at this state concerning solubility; *i.e.* low room temperature may result in disadvantageous precipitation because of the concentration of the solution being too near the saturation point.

Procedure—The procedure is exactly the same as for determining Ca in serum, except the use of 0.4 ml. of 4 per cent ammonium oxalate and 0.1 ml. of serum. Place this dilution of serum in a test tube, mix them immediately, and knock the bottom of the test tube sharply several times with the tip of the finger to make the solution bubble (room temperature, 20°-25°). After 3 minutes add 2 ml. of reagent (A) and 2.5 ml. of reagent (B). Read the photometric scale for the sample against the reagent blank set at 30 per cent transmission with a 620 mμ-filter. The blank solution used previously in the Ca determination can be used here, although there is some possibility of contamination with oxalic acid; hence, it is advisable to use a new solution instead: *i.e.* 0.4 ml. of 4 per cent ammonium oxalate solution, 0.1 ml. of distilled water, 2 ml. of reagent (A) and 2.5 ml. of reagent (B). The photometric reading given above determines the content of compound Ca in the serum.

Calculation—The total value of Ca in the serum is determined as follows:

Determine the Ca value in the serum as described in *Section I*; denote as (1). Calculate the value of compound Ca in the serum from the standard curve as described in

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* The standard curve plotted on a semi-log paper shows a curvature of the line above 12 γ of Ca. This is explained by the fact that two particles of dye usually adhere to one particle of Ca, but Ca in excess converts this dye-Ca complex (2 moles to 1 mole) to one stain one Ca complex. Although usual serum contains compound Ca which forms a one to one combination with the dye, it is also known that the readings for such a combination gives a straight line up to about 15 γ. For any reading a little beyond 12 γ, therefore, an appropriate Ca value is still obtainable on the same but extended line of the standard curve (*cf.* Fig. 1).

Section II; denote as (II). Then, the dialytic Ca in the serum is given by the relation: Dialytic Ca in serum=(I)–(II). The average content of dialytic Ca will be 4–5 γ per 0.1 ml. of serum.

III. Determination of Mg in Serum

Exactly the same process is followed for determining Mg in serum as for determining Ca. The use of 0.2 *N* and 10 *N* sodium hydroxyde solutions instead of 2 *N* sodium hydroxyde solution is the only difference. By this procedure, Ca and Mg are determined simultaneously.

Reagent—

A) 0.00025 *M* disodium 1-hydroxy-4-chloro-2,2-diazobenzene-1,8-dihydroxy-naphthalene-3, 6-disulfonic acid.

B) Sodium hydroxide, volumetric 0.2 *N*.

C) Sodium hydroxide, volumetric 10 *N*.

D) Calcium standard solution, (Ca in 20 γ per 1 ml.)

E) Magnesium standard solution: Dilute 10 mg. of powdered metallic magnesium in a 100 ml.—volumetric flask with 1.0 ml. of concentrated hydrochloric acid; shake until dissolved completely; dilute to 100 ml. with distilled water. To 10 ml. of this solution add distilled water to 100 ml. Then each ml. of the resulting solution contains 10 γ of Mg.

Standard Curve—Place 0, 0.1, 0.2, 0.3, 0.4, and 0.5 ml. of Ca standard solution into 6 test tubes respectively; dilute the content of each test tube with distilled water to 0.5 ml.; add 2 ml. of reagent (A) and 2.5 ml. of reagent (B). Three minutes later,

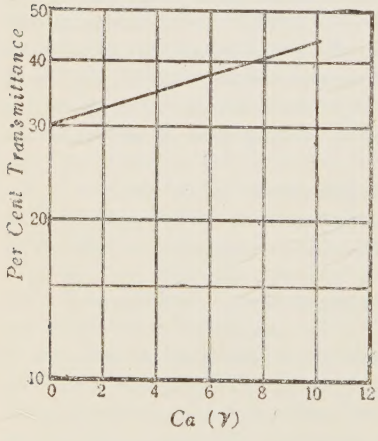

Fig. 1. Standard curve of Ca.

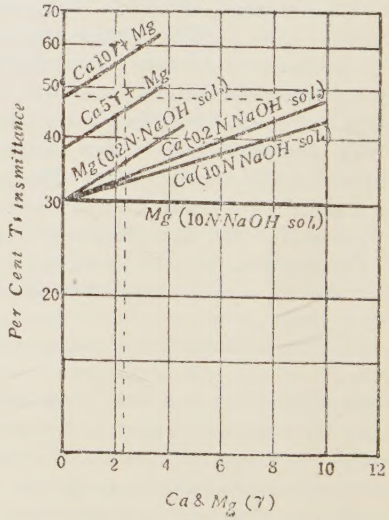

Fig. 2. Standard curve of Ca and Mg.

read the calibration of the electrophotometer with a 620 mμ-filter after setting the reading for the blank solution of zero concentration at 30 per cent transmission. As the second step, add 0.5 ml. of 10 *N* sodium hydroxide solution into each test tube and mix. After a while, read the scales in the same manner. Draw two standard curves for Ca as shown in Fig. 2, the one for 0.2 *N* and another for 10 *N* sodium hydroxyde solution.

The same method is followed for determining Mg. Use the standard test solution of Mg (Reag. E) (*loc. cit.*). Draw corresponding standard curve (linear up to 6 γ of Mg). It has already been shown that Mg makes no contribution to light transmission if 10 *N* sodium hydroxide solution is added, regardless of the amount of Mg present. Since the photometric readings for a solution containing 4.1 γ of Ca are the same as those for a solution containing 2.5 γ of Mg, it is not necessary to draw a standard Mg curve empirically.

Procedure—Place 0.4 ml. of distilled water into a clean test tube and add 0.1 ml. of fresh non-hemolyzed serum; clean the pipette thoroughly; add 2 ml. of reagent (A) and mix well; after a short while, add 2.5 ml. of reagent (B). For the blank solution, add 2 ml. of reagent (A) and 2.5 ml. of reagent (B) to 0.5 ml. of distilled water. Place these mixtures into cuvettes. Exactly 3 minutes after mixing reagent (B), read the scale for the test serum solution setting the scale for the blank solution at 30 per cent transmission with a 620 mμ-filter. The above gives the reading for Ca plus Mg.

As the second step, we have to eliminate the Mg reading. For this purpose, add 0.5 ml. of reagent (C) (10 *N* NaOH) to the content of each tube and mix well. After 5 minutes, read the scale as before. The reading gives the amount of Ca independently of Mg, since Mg forms $Mg(OH)_2$ and does not affect the reading.

Calculation—Having followed the latter procedure, the amounts of Ca and Mg can now be determined simultaneously from the standard curves. For example, if a reading of 56 per cent for the solution in the first step and a reading of 43.5 per cent for the solution of the second step were obtained, the determination of Ca and Mg would proceed as follows: Follow the Ca (10 *N* NaOH) standard line to the point whose ordinate is 43.5; then, the abscissa of this point is read to be 10 γ; this is the amount of Ca desired. On the Ca (0.2 *N* NaOH) standard line, take the point whose abscissa is 10 γ; then, the ordinate of this point is read to be 47.5; from the point on the vertical axis reading 47.5, draw a parallel line to the Mg standard line; follow this line to the point whose ordinate is 56; then, the abscissa for the point is read to be 2.2 γ; this is the amount of Mg derived. If the Ca content of serum is known to vary between 14 and 17 mg., as in case of rabbits, it is advisable to use 0.05 ml. of serum, or to use twice the amount of reagent (A) and (B) as before, if the same degree of accuracy is desired.

IV. Determination of Dialytic Mg in Serum

Reagents—The same as for the previous determination of Mg.

Procedure—The determination of dialytic Mg in serum is an extension of the preceding method. The difference is with respect to the order in which the reagents are added.

Place 0.4 ml. of 0.2 *N* sodium hydroxide solution into a test tube and add 0.1 ml. of serum. Mix while tapping sharply the bottom of the test tube to ause bubbling (20°–25°). Exactly 3 minutes later add 2.0 ml. of reagent (A) and mix. Add 0.4 ml. of distilled water and 2.1 ml. of reagent (B). Another 3 minutes later read the scale of the serum tube comparing to the blank solution used in the determination of Mg. This will give the total amount of compound Mg plus total Ca. Proceed carefully so as to eliminate possible errors arising from the room temperature at the time of mixing.

Calculation—The compound Mg is determined from the Mg standard curve in the usual manner. Subtracting the compound Mg from the total Mg in serum, gives the amount of dialytic Mg.

V. Determination of Ca and Mg in Water

We can determine the hardness of water by application of the previous methods for determination of Mg in serum. In this case, 0.5 ml. of the water to be tested is used instead of 0.1 ml. of serum; add 0.4 ml. of distilled water; determine the amount of Ca and Mg as before. The total hardness of water is expressed by number following the formulae:

$$\text{Water hardness: } CaCO_3(\text{p.p.m.}) = Ca(\text{p.p.m.}) \times 2.497 + Mg\ (\text{p.p.m.}) \times 4.115$$

when p.p.m.=mg./*l.* of water, *cf.* Fig. 3. For a gross determination of total hardness, the process can be shortened by using only the reading for the 0.2 *N* sodium hydroxide solution. The colouring relation of color development between Mg and Ca, *i.e.* the photometric reading for 4.115 γ of Ca is the same as for 2.497 γ of Mg, and the proportion remains the same. It is suggested, therefore, that a standard curve may be drawn for the hardness of water as shown in the diagram, and then determine the hardness of water correspondingly. When the Ca content is known to be more than 10 γ, it is recommended that the testing water be diluted, *e.g.* decrease the quantity of test water to 0.25 ml. or 0.1 ml.

VI. Minimal Determination of Water Hardness

For softening hard water, successive tests are required until the total hardness is lowered to 0.2 p.p.m. of $CaCO_3$. The method allows minimal determination of water hardness down to 0.01 p.p.m.

Reagents—

A) 0.00025 *M* disodium 1-hydroxy-4-chloro-2,2-diazobenzene-1,8-dihydroxy-naphthalene-3,6-disulfonic acid.

B) Alkaline buffer solution: Take 1.0 g. of sodium diethyl barbiturate ($C_8H_{11}O_3N_2Na$) and 15 ml. of 0.1 *N* sodium hydroxide solution; dilute with distilled water to 100 ml.*

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* It is neccessary to adjust the amount of 0.1 *N* sodium hydroxide solution so as to obtain equivalent photometric readings for both Mg 2.5 γ and Ca 4.1 γ.

C) Standard solution: Make two solutions, one containing 1 γ of Ca and the other 1 γ of Mg for 5 ml. of solution.

Standard Curve—Place 0, 1, 2, 3, 4, and 5 ml. of Ca standard solution into 6 test tubes, and dilute the content of each test tube to 5 ml. with distilled water: then 0, 0.2, 0.4, 0.6, 0.8 and 1.0 γ of Ca are contained in these test tubes, respectively. Add 1 ml. each of reagents (A) and (B) into each test tube. Transfer contents into cuvette; exactly 3 minutes after adding reagent (B) read the photometric scales setting the reading for the blank solution at 50 per cent transmission with a 620 mμ-filter.

For Mg, the same procedure is followed. Plot these two series of readings as shown in Fig. 4. Now 0.41 γ of Ca is supposed to have the same galvanometric readings as does 0.25 γ of Mg. If the Ca reading is less than expected, increase the 0.1 *N* sodium hydroxide alkaline buffer solution to 16 ml. or to 17 ml. If Ca is higher than expected, decrease to 14 ml. or 13 ml.—in this way it is possible to plot the standard curve as shown in Fig. 4. Any unbalance results from differences in the filters used.

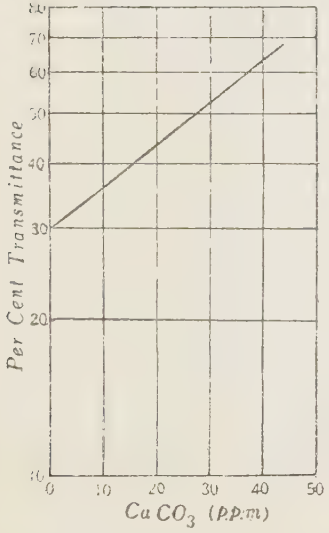

Fig. 3. Standard curve of hardness of water

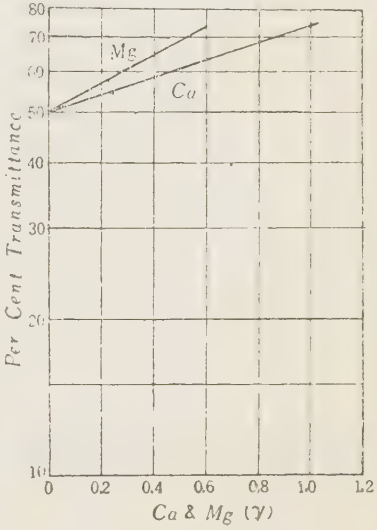

Fig. 4. Standard curve of Ca and Mg. (Super-micro method)

Procedure—Place 5 ml. of test water into a test tube; add 1 ml. each of reagents (A) and (B). Make a blank solution by adding the same amount of reagent (A) and (B) to 5 ml. of distilled water. Exactly 3 minutes after adding reagent (B), read the scale for the test water, setting the blank solution reading at 50 per cent transmission point with a 620 mμ-filter. If extraneous substances, *e.g.*, Fe, Co, Ni, and Mn are present, the following procedure is devised (see next *Section VII*). Greater accuracy can

be obtained by adjusting from alkaline toward neutral.

Calculation—The water hardness is calsutated from the standard curves in Fig. 4. The method is sensitive to 0.01 p.p.m. of $CaCO_3$.

VII. Determination of Ca and Mg in Mineral Water

If the water contains additional minerals, *e.g.*, Fe, Co, Ni, Mn, in excess of one-fifth the amount of Ca or Mg present, the method is no longer applicable. Such mineral water must be treated as follows: Add 0.5 ml. of 1 per cent sodium cyanide solution to 0.5 ml. of test water; mix well; heat for ten minutes at 40–50° shaking occasionally. Any precipitate should be removed by centrifugation. Filtration of the water is not satisfactory because small amounts of Ca and Mg from the filter-paper might be introduced. Take 0.5 ml. of this solution and determine the water hardness as previously. The procedure is satisfactory, even if Fe, Co, Mn or Ni is known to be present by as much as ten times the amount of Ca or Mg present. Since sodium cyanide solution has no known effect on photometric determinations, simple distilled water with reagents may also be used for the blank solution *c.f.* Mg determination.*

VIII. Determination of Ca and Mg in Food

Ca and Mg in food can also be determined by the present method. Take 1–5 g. of food in a crucible; add a small amount of 10 per cent acetic acid solution and heat over a Bunsen-burner. Heat slowly at the start; after evaporation of the acetic acid, heat strongly until the bottom becomes red hot. Burn the residues to white ash; weigh these ashes. Repeat heating and weighing until the weight becomes constant. Add a small amount of 2 *N* hydrochloric acid solution to the ash and dissolve. Dilute further with distilled water until the content of Ca and Mg in 0.25 ml. of solution is less than 10 γ. Take 0.5 ml. of the solution and add 0.5 ml. of a 1 per cent solution of sodium cyanide as used previously for the determination of mineral water hardness. Heat for 10 minutes at 40–50° (the solution must be alkaline). Take 0.5 ml. of this solution; determine the Ca and Mg by the methods used in the determination of Mg. If the amount of Ca and Mg are observed to be more than 12 γ and 6 γ respectively, dilute further and repeat the determination.**

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* If the mineral water is strongly acid, as indicated by the red colour after adding reagent (A), add ammonium hydroxide until the colour changes to purple (reagent (A) turns purple at pH 7, thereby giving a rough measure of the pH of the test water). If large amounts of Cd^{++} or Zn^{++} are present, the Mg determination is inaccurate, but the Ca determination is accurate.

** Test food must be collected equally from every part. Corn must be ground, vegetables must be washed, dried naturally in one day, and weighed.

If the ash does not become completely white, cool the crucible; add a small amount of distilled water; heat in a water bath until evaporation is completed; then heat directly over a gas flame as before.

SUMMARY

The author presented a method for colorimetric micro-determination of Ca and Mg. This direct method is very simple and direct as well as very sensitive. Total Ca and Mg as well as dialytic Ca and Mg in serum, especially the latters which are difficult to be determined by other methods, can be determined with comparative simplicity. Water hardness, and Ca and Mg in food can also be determined by an extension of the proposed method.

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The Journal of Biochemistry, Vol. 42, No. 1, 1955

THE INCORPORATION OF PHOSPHORUS FROM P^{32}-LABELED COCARBOXYLASE INTO ADENOSINE TRIPHOSPHATE BY RAT LIVER HOMOGENATE AND CYCLOPHORASE SYSTEM

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(Received for publication, October 4, 1954)

It is well known that the decarboxylic oxidation of pyruvate and α-ketoglutarate in animal tissues requires cocarboxylase as a coenzyme (*1–5*), but the role of this coenzyme is not perfectly clear. In the previous paper, Ogata and Hashimoto (*5*) reported that the KCl isotonic homogenates of liver and kidney from thiamine deficient rats showed in the presence of α-ketoglutarate a lower rate of oxidation and coupled phosphorylation than those of the normal or pair-fed control rats, and that the mixture of catalytic amounts of cocarboxylase and thiamine restored the oxidation and coupled phosphorylation to some extent. In the later experiments (*6*) it was further demonstrated that thiamine-deficient rats showed a significant decrease in the turnover rate of labile P of ATP and ADP as compared with normal and pair-fed control rats one hour after the intraperitoneal injection of inorganic P^{32}, and this decrease was restored to the normal range in recovered rats. To obtain further evidence on the precise role of cocarboxylase in the oxidative phosphorylation of α-ketoglutarate and pyruvate incubated with tissue suspension, the rate of incorporation of the phosphorus of P^{32}-labeled cocarboxylase (P^{32}-cocarboxylase) into the terminal P of APT was investigated with a rat liver homogenate or cyclophorase system.

EXPERIMENTAL

Preparation of Reaction Components—P^{32}-labeled cocarboxylase was synthesized by the method of Tauber (*7*) for use in the homogenate experiment. The preparations used in the experiment were found to be of more than 90 per cent purity by the measurement of the absorption at 260 mμ and the phosphorus content, but this synthetic method was rather difficult, and the phosphorus content of synthetic material was often found

to be far lower than the theoretical value. In the later experiments using liver cyclophorase system, the method of Viscontini, Bonetti, and Karrer (*8*) was enployed. Cocarboxylase prepared by this method was contaminated to a small extent by thiamine triphosphate which was shown by the determination of acid-labile P and total P of the preparations, but it was very difficult to remove this contamination, as pointed out in the original paper. The specific activity of the P^{32}-cocarboxylase used in the experiments was of the order of 200,000–300,000 counts per minute per 2 mg., which were added to the reaction mixture. The contaminated inorganic P^{32} was always lower than 2 per cent of the total P^{32}. ATP was prepared from rabbit muscle in the form of barium salt by the method of Dounce and others (*9*), and this preparation was found to contain about 10 per cent of ADP and negligible amount of AMP, by the chromatographic analysis of Cohn and Carter (*10*).

ATP-ase was isolated from rabbit muscle by the method of Bailey (*11*) and later, by that of Szent-Györgyi (*12*), and the latter preparation was further purified employing the method of Bailey (*11*).

Treatment of Animls—Rats weighing 100–150 g. were used in the experiments. They received a nutritionally complete synthetic ration as described in the previous paper (*5*,*6*). The thiamine-deficient rats received the thiamine-free synthetic ration, and were used for the experiment when a marked deficiency symptoms appeared.

Tissue Preparations—Rat liver homogenate and cyclophorase system (R_2L or R_3L) were used in the experiments. Isotonic liver homogenate was prepared as described in the previous paper (*5*), and 1 ml. of 20 per cent liver homogenate was placed in each main compartment of the Warburg vessel. The liver cyclophorase system was prepared by the method of Green (*13*) and Lipmann (*15*). R_2L or R_3L prepared from 5 g. of rat liver was diluted to 10 ml. by the addition of 0.9 per cent KCl containing 0.02 *M* $NaHCO_3$, and 0.7 ml. or 1 ml. of his suspension was placed in each Warburg vessel.

Reaction Mixture—In the experiment using homogenate, each manometer vessel contained: 1 ml. of 0.04 *M* substrate solution (Na α-ketoglutarate, Na pyruvate, or succinate), 1 ml. of 0.008 *M* K ATP, 0.4 ml. of 0.06 *M* K phosphate buffer (pH 7.4), 0.8 ml. of 0.5 *M* KCl, 0.4 ml. of 0.1 *M* $MgCl_2$, 1 ml. of P^{32}-cocarboxylase solution (2 mg. of P^{32}-cocarboxylase), and enough water added to make the total volume of 6 ml. Just before the measurement of oxygen uptake, 1 ml. of 20 per cent liver homogenate was added to the system. In the control experiments carrier-free inorganic P^{32} (as Na_2HPO_4) contained in 0.5 ml. of 0.06 *M* K phosphate buffer was used instead of P^{32}-cocarboxylase.

This concentration of phosphate buffer in the control experiments was chosen so as to make the concentration of inorganic P the same as in the main experiments after incubation with the homogenate, because a greater part of added cocarboxylase was found to be rapidly decomposed by the tissue homogenate, as will be described later.

Each center cup of the Warburg vessel contained 0.2 ml. of 2 *N* KOH and a folded filter paper.

The gas phase was air. The incubation was carried out by shaking at 25°.

The reaction mixture of the experiment with liver cyclophorase consisted of the following components: 1.0 ml. of 0.04 *M* substrate solution (α-ketoglutarate, pyruvate, or succinate), 0.2 ml. of 0.1 *M* $MgCl_2$, 0.4 ,ml of 0.06 *M* K phosphate buffer (pH 7.4), 1.0 ml. of 0.008 *M* K ATP, 1.0 ml. of P^{32}-cocarboxylase solution (2 mg.), and enough water added to make a total volume of 5 ml. In the control experiments 0.1 ml. of carrier-free inorganic P^{32} and the same amount of unlabeled cocarboxylase were added to the system. Just before the measurements of oxygen uptake, 0.7 ml. of R_2L or R_3L was added to the system. The incubation was carried out at 25°.

The reaction mixture of the "gel P" (*12*) consisted of the following components: 1.0 ml. of 0.1 *M* α-ketoglutarate, 0.2 ml. of 0.1 *M* $MgCl_2$, 0.4 ml. of 0.06 *M* k phosphate buffer, 0.8 ml. of 0.5 *M* KCl, 0.5 ml. of 0.008 *M* ATP, 1.0 ml. of P^{32}-cocarboxylase (2 mg.) or 0.1 ml. of inorganic P^{32}, enough water added to make the final volume to 5 ml. Just before the measurement of oxygen uptake, 1.0 ml. of R_2L was added to the reaction mixture. The gas phase was air.

Analyses—The reaction in each vessel was stopped at a suitable point by transfer of the vessel into an ice bath and addition of 2 ml. of 30 per cent trichloroacetic acid. The contents of two Warburg vessels were combined and centrifuged. The precipitate was washed once with 4 ml. of 5 per cent trichloroacetic acid. The trichloroacetic acid extract was carefully neutralized to phenolphtalein first with 20 per cent NaOH and then with 2 *N* NaOH in a cold room. One ml. of 1 *M* acetic acid was added and ATP was precipitated by the addition of 20 per cent mercuric acetate solution. The mixture was placed in a cold room overnight and centrifuged. An aliquot of the supernatant was used for the measurement of the specific activity of inorganic P. The precipitate was washed with 5 per cent mercuric acetate solution and suspended in 4 ml. of ice water. Mercury was removed by passing hydrogen sulfide through the suspension in an ice bath for 10 minutes. The mixture was centrifuged and the precipitate was washed with 2 ml. of water. The combined extracts were aerated in the cold for one hour to remove hydrogen sulfide, made up to 6 ml., and 2 ml. of this nucleotide solution was then used for the measurement of the specific activity of the terminal P of ATP.

For the determination of the specific activity of the terminal P of ATP in nuclotides insoluble in mercuric acetate, a reaction mixture used consisted of 1.5 ml. of APTase solution, 1.5 ml. of 0.1 *M* glycine-NaOH buffer (pH 9.0), 0.1 ml. of 0.18 *M* $CaCl_2$, and 2 ml. of the nucleotide solution. After incubation for 30 minutes at 37°, 3 ml. of 30 per cent trichloroacetic acid solution was added to the reaction mixture and filtered. An aliquot of the filtrate was used for the determination of inorganic P^{31} and P^{32} in the reaction mixture, as described below. In the control experiment, which was necessary in order to measure the contaminated inorganic P^{32} in the mercuric acetate precipitate, 2 ml. of water was added to the reaction mixture instead of the ATPase solution and analyzed as described above. This value was corrected for the main experiment.

For the determination of inorganic P^{31} in the reaction mixture, Fiske-Subbarow's (*16*) method was employed, and for the determination of inorganic P^{32}, iso-

butanol method of Enner (*17*) was employed.

Analysis of Gel P—The reaction was stopped at varying time point by transfer of the reaction mixture into 30 ml. of ice-cold 0.9 per cent KCl solution. After washing the precipitate twice with ice-cold 0.9 per cent KCl solution through centrifugation, the precipitated gel was suspended in water, deproteinized with trichloroacetic acid, diluted with water to a definite volume, and an aliquot was used for the determination of inorganic P and gel $\Delta 7$ P. Inorganic P^{31} was deterined by employing the method of Shinowara, Jones, and Reinhart (*18*), and P^{32} by the method of Enner (*17*). Gel $\Delta 7$ P was determined as inorganic P after heating with 1 N H_2SO_4 for 15 minutes in a boiling water bath.

RESULTS

Experiments with Rat Liver Homogenates

In a preliminary experiment, it was shown that when P^{32}-labeled cocarboxylase was incubated for 20 minutes at 32° in the presence of α-ketoglutarate, pyruvate, or succinate, P^{32} of labeled cocarboxylase was rapidly incorporated into the terminal P of ATP in an aerobic condition, while the incorporation was very slow in an anaerobic condition. In the present experiment, however, the decomposition rate of the terminal P of cocarboxylase by tissue homogenate was very high and it was difficult to distinguish the route by which P^{32} of cocarboxylase is incorporated directly (not via inorganic P) into ATP from the route by which inorganic P^{32} is released from P^{32}-cocarboxylase and then incorporated into ATP by oxidative phosphorylation. To ascertain the existence of the latter route, the incubation was carried out at a lower temperature (25°) and the relative specific activity (R.S.A.)* of the terminal P of ATP incubated with P^{32}-cocarboxylase for various incubation periods was compared with that incubated with inorganic P^{32}, paying special attention to the earlier stages of the incubation. The results are presented in Table I. As shown in the table, R.S.A. of terminal P of ATP incubated with P^{32}-cocarboxylase was greater than that incubated with inorganic P^{32} at each incubation time when α-ketoglutarate or pyruvate was used as the substrate and such results were not obtained in the presence of succinate. The results indicated that there might be some route by which cocarboxylase-P transfers directly (not *via* inorganic P) to the terminal P of ATP, coupled with the oxidation of α-ketoglutarate and pyruvate by the tissue homogenates. However, definite results were not obtained by these homogenate experiments, as cocarboxylase was decomposed rapidly by tissue homogenates even at 25°. To prevent this decomposition of cocarboxylase by tissue homogenates, which may be expected by tissue phosphatase, experiments with liver cyclophorase system were carried out, as described in the next section.

Experiments with Liver Cyclophorase System

Using the liver cyclophorase system, it was possible to remove a majority of the

* See *Foot note* on Table I.

TABLE I

Change in R.S.A. of the Terminal P of ATP Occurring on Incubation of P^{32}-Cocarboxylase or Inorganic P with Rat Liver Homogenates*

1) Substrate: α-Ketoglutarate

Addition \ Incubation time *min.*	2	5	10	15
P^{32}-Cocarboxylase		80	95	106
Inorganic P^{32}	41	56	80	88

2) Substrate: Pyruvate

Addition \ Incubation time *min.*	2	5
P^{32}-Cocarboxylase	24	59
Inorganic P^{32}	18	43

3) Substrate: Succinate

Addition \ Incubation time *min.*	2	5	30
P^{32}-Cocarboxylase	28	40	57
Inorganic P^{32}	31	43	72

The incubation mixture contained 1 ml. of 0.04 *M* substrate solution, 1 ml. of 0.008 *M* K-ATP, 0.4 ml. of 0.06 *M* K phosphate buffer (pH 7.4), 0.8 ml. of 0.5 *M* KCl, 0.4 ml. of 0.1 *M* $MgCl_2$, 2 mg. of P^{32}-cocarboxylase. Final volume, 6 ml. In the control experiment 0.5 ml. of 0.06 *M* K phosphate buffer and carrier-free inorganic P^{32} were added. Incubation at 25°, in air.

* $\text{R.S.A.} = \frac{\text{specific activity of the terminal P of ATP}}{\text{specific activity of the medium inorg. P}} \times 100$

activity of tissue phosphatase and a more lucid results could be obtained. As shown in Table II, when P^{32}-cocarboxylase was incubated with R_3L in the presence of α-ketoglutarate or pyruvate as the substrate, R.S.A. of the terminal P of ATP increased prog-

TABLE II

Change in R.S.A. of the Terminal P of ATP Occurring on Incubation of P^{32}-Cocarboxylase or Inorganic P^{32} Plus Unlabeled Cocarboxylase with Rat Liver R_3L*

1) Substrate: α-Ketoglutarate

Addition \ Incubation time *min.*	2	5	15
P^{32}-Cocarboxylase	45	89	228
Inorganic P^{32}+unlabeled cocarboxylase	7	22	76

2) Substrate: Pyruvate

Addition \ Incubation time *min.*	5	10	20
P^{32}-Cocarboxylase	83	141	190
Inorganic P^{32}+unlabeled cocarboxylase	32	56	70

3) Substrate: Succinate

Addition \ Incubation time *min.*	5	15
P^{32}-Cocarboxylase	71.5	102
Inorganic P^{32}+unlabeled cocarboxylase	46	84

Incubation mixture: 1.0 ml. of 0.04 *M* substrate solution (α-ketoglutarate, pyruvate, or succinate), 0.2 ml. of 0.1 *M* $MgCl_2$, 0.4 ml. of 0.06 *M* K phosphate buffer (pH 7.4), 1.0 ml. of 0.008 *M* K ATP, 1.0 ml. of P^{32}-cocarboxylase solution (2 mg.); final volume 6 ml. In the control experiment, carrier-free inorganic P^{32} and 2 mg. of unlabeled cocarboxylase were added to the system. Incubation at 25°, in air.

* See *Foot note* on Table I.

ressively as the reaction proceeded and finally reached a value above 100. In the control experiments, in which carrier-free inorganic P^{32} and unlabeled cocarboxylase were incubated with R_3L, R.S.A. of the terminal P of ATP at each incubation time was lower than that of the main experiment and always below 100 by 15–20 minutes' incubation. These observations led to the conclusion that some route might exist, by which P^{32} of cocarboxylase is incorporated directly (not *via* inorganic P) into the terminal

P of ATP when incubated with the liver cyclophorase system in the presence of α-ketoglutarate or pyruvate. Such an explicit result was not obtained when succinate was used as the substrate, as shown in Table II (*3*).

It was expected from our previous experiments (*5*, *6*) that a more lucid results might be obtained by using the cyclophorase system of thiamine-deficient rats. Therefore, using R_2L prepared from thiamine-deficient rats, similar experiments were carried out. As shown in Table III, a more marked difference was observed between R.S.A. of the terminal P in the main experiment, where P^{32}-cocarboxylase was added, and that in the control experiment, where inorganic P^{32} was added, than that obtained in the experiment using the normal rat liver cyclophorase system.

TABLE III

Incorporation of P of Labeled Cocarboxylase into ATP Incubated for 10 Minutes at 25° with the Cyclophorase System (R_2L) of Thiamine-Deficient Rat

Experimental No.	Substrate	Addition	R.S.A.* of the terminal P of ATP
1	α-Ketoglutarate	P^{32}-Cocarboxylase	367
		Unlabeled cocarboxylase + inorganic P^{32}	38
	Pyruvate	P^{32}-Cocarboxylase	250
		Unlabeled cocarbyxylase + inorganic P^{32}	34
	Succinate	P^{32}-Cocarboxylase	184
		Unlabeled cocarboxylase + inorganic P^{32}	81
11	α-Ketoglutarate	P^{32}-Cocarboxylase	152
		Unlabeled cocarboxylase + inorganic P^{32}	20
		Inorganic P^{32}	38
	Pyruvate	P^{32}-Cocarboxylase	112
		Unlabeled cocarboxylase + inorganic P^{32}	32

The experimental conditions were the same as those in Table II.

* See *Foot note* on Table I.

It was found that NaF added to the incubation mixture almost completely prevented the release of inorganic P from cocarboxylase. Therefore NaF was added to the system and the approximately rate of incorporation of P^{32} of labeled cocarboxylase into ATP was compared with the rate of that of inorganic P^{32}. In this case the specific activity of added P^{32} cocarboxylase and that of inorganic P^{32} were corrected to the same value *i.e.* 100, and

$$\left[\frac{\text{specific activity of the terminal P of ATP}}{\text{specific activity of the added cocarboxylase}}\right] \text{ was compared with } \left[\frac{\text{specific activity of the terminal P of ATP}}{\text{specific activity of the added inorganic P}}\right].$$

The results, shown in Table IV, indicate that the incorporation rate of P^{32}-cocarboxylase into ATP is about one-tenth of that of inorganic P^{32}.

TABLE IV

*Comparison of the Approximate Rate of Incorporation of P^{32}-Cocarboxylase into ATP with That of Inorganic P^{32} in Liver Cyclophorase System (R_2L) in the Presence of α-Ketoglutarate**

Addition	R.S.A.†	
	5 minutes	10 minutes
P^{32}-Cocarboxylase	3.9	6.6
Inorganic P^{32}	50	68

$$\text{R.S.A.}\dagger = \frac{\text{specific activity of the terminal P of ATP}}{\text{specific activity of the added } P^{32}\text{-cocarboxylase of inorg. } P^{32}}$$

* The final concentration of $5\times10^{-5}\,M$ NaF was added to the system. Other experimental conditions were the same as those in Table II.

Next, various amounts of P^{32}-cocarboxylase were added to the system and the radioactivity of the terminal P of ATP was compared in each case. The results represented in Table V showed that the radio-activity of the terminal P of ATP increased in proportion to the increased amount of added cocarboxylase, up to 2 mg. of cocarboxylase, which was rather a large amount.

This result indicates that in the first step of the incorporation, P^{32}-cocarboxylase may exchange with the cocarboxylase in the reaction center (which may consist of lipothiamide pyrophosphate according to Reed *et al.* (*22, 23*) and then transfer to ATP, as will be discussed later.

The rate of incorporation of the phosphorus P^{32} of P^{32}-cocarboxylase into ATP was not inhibited by 2,4-dinitrophenol (final concentration $1\times10^{-4}\,M$), which inhibited the incorporation of inorganic P into ATP, as shown in Table VI.

TABLE V

Change in Radioactivity of the Terminal P of ATP when Various Amount of P^{32}-Cocarboxylase was Incubated with Liver Cyclophorase System (R_2L) in the Presence of α-Ketoglutarate

(Radioactivity of ATP in the presence of 2000 γ of cocarboxylase was taken as 100)

Addition		NaF* (+)	NaF (−)
Incubation time	*min.*	10	5
Amounts of cocarboxylase	2000 γ	100	100
	1000	44	62
	400	20	23
	100	(10)	(9)

The experimental conditions were the same as those in Table II.

* Final concentration of NaF: 5×10^{-5} *M*.

TABLE VI

Effect of 2,4-Dinitrophenol (DNP) on the Incorporation of P of Cocarbocylase or Inorganic P into ATP Incubated with Liver Cyclophorase System (R_2L) in the Presence of α-Ketoglutarate

Addition	Incubotion time	R.S.A.* of the terminal P of ATP	
		DNP (+)	DNP (−)
P^{32}-Cocarboxylase	5 *min.*	265	202
	10	550	585
Unlabeled cocarboxylase +inorganic P^{32}	5	10	57
Inorganic P^{32}	5	13	106

* See *Foot note* on Table I.

The final concentration of 2, 4-dinitrophenol was 1×10^{-4} *M*.

Other experimental conditions were the same as those in Table II.

Experiment with 'Gel'-P—The specific activities of gel-Δ7 P and gel P were compared with the specific activity of medium inorganic P, and the data are shown in Table VII. In this case, the specific activity of gel Δ7 P was greater than that of the medium inorganic P or gel P when P^{32}-cocarboxylase was incubated with R_2L at 25° for 20

TABLE VII

Relative Specific Activity of Gel-P and Gel-Δ7P Incubated with P^{32}-Cocarboxylase or Inorganic P^{32} with R_2L in the Presence of α-Ketoglutarate

Temperature	Incubation time	Addition			
		P^{32}-Cocarboxylase		Inorganic P^{32}	
		Gel-P	Gel-Δ7P	Gel-P	Gel-Δ7P
25°	20 min.	95*† (5.0)	550† (18.0)	84† (60)	110† (70)
0°	30	20† (0.4)	160† (3.6)		

* The mean value of three experiments.

† $\text{R.S.A.} = \frac{\text{specific activity of gel P or gel } \Delta 7\text{P}}{\text{specific activity of medium inorganic P}} \times 100$

The figures in parentheses represents:

$\text{R.S.A.} = \frac{\text{specific activity of gel P or gel } \Delta 7\text{P}}{\text{specific activity of the added } P^{32}\text{-cocarboxylase or inorgnic } P^{32}} \times 100$

Incubation mixture: 1.0 ml. of 0.1 *M* α-ketoglutarate, 0.2 ml. of 0.1 *M* $MgCl_2$, 0.4 ml. of 0.06 *M* K phosphate buffer, 0.8 ml. of 0.5 *M* KCl, 0.5 ml. of 0.008 *M* ATP, 1.0 ml. of P^{32}-cocarboxylase (2 mg.) or 0.1 ml. of inorganic P^{32}, 1.0 ml. of R_2L. Final volume, 5 ml.

Incubation at 25°, in air.

minutes or at 0°for 30 minutes. On the contrary, rapid equilibrium was established between the specific activities of gel P and gel Δ7P when inorganic P^{32} was added to the system. These results indicate that cocarboxylase appears as labile P in the mitochondrial system.

DISCUSSION

There have been only few reports concerning the precise role of cocarboxylase in the oxidation or especially oxidative phosphorylation of α-ketoglutarate and pyruvate in the animal tissues. Kissling and Lindahl (*19*) pointed out already that inorganic P^{32} was taken up into the cocarboxylase in the mitochondrial suspension and there was a fine "proportionality" between the rate of incorporation and metabolic rate, measured as oxygen consumption where pyruvate was the substrate. Recently, however, Bartley (*20*) reported that incubation of

respiratory mitochondria with cocarboxylase and P^{32}-labeled orthophosphate led to the appearance of a labeled phosphate, but the incorporation was very slight and was unrelated to the amount of substrate oxidized. His experimental results were remarkable in that the specific activity of thiamine monophosphate showed a relatively high value and α-ketoglutarate caused a more rapid incorporation of P than did succinate or in the absence of a substrate. Therefore, he postulated the following schema.

(1) Cocarboxylase+α-lipoic acid⟶lipothiamide pyrophosphate
(2) Lipothiamide pyrophosphate⟶lipothiamide+2 orthophosphate
(3) Lipothiamide+orthophosphate or ATP⟶lipothiamide monophosphate
(4) Lipothiamide monophosphate⟶α-lipoic acid+thiamine monophosphate

In our experiments, it was shown that there was a fine proportionality between the specific activity of the terminal P of ATP and the amount of P^{32}-cocarboxylase (up to 2 mg.). The results suggest that the binding of cocarboxylase with the enzymatic center, which may consist of lipothiamide according to Reed *et al.*, (*22*, *23*) may be an active intermediate of the phosphate transfer from cocarboxylase to ATP in the presence of α-ketoglutarate or pyruvate plus fumarate.

Therefore the incorporation of P^{32} of cocarboxylase into ATP may be formulated as follows, considering the terminal phosphate bond of cocarboxylase is a high energy phosphate bond.

(1) Cocarboxylase+α-lipoic acid⟶lipothamide pyrophosphate
(2) Lipothiamide pyrophosphate+ADP⟶ATP+lipothiamide monophosphate

In Bartley's experiment, it is rather inprobable that the total amount of the added cocarboxylase can bind with the enzymatic center or α-lipoic acid and become an active form.

Taking these considerations into account, the rate of incorporation of phosphorus from active form of cocarboxylase (lipothiamide pyrophosphate) into ATP might be more rapid than the rate of incorporation of phosphorus from added cocarboxylase into ATP, which was measured in the present experiments.

Further, it was shown that this phosphate transfer reaction was not inhibited by 2,4-dinitrophenol. In this respect, the behavior of cocarboxylase is different from that of inorganic P in its phosphate incorporation into ATP.

As for the 'gel P', which was proposed by Green *et al.* (*21*) as the intermediate prior to ATP in the oxidative phosphorylation, it is

shown that 'gel P' may not be the precursor of ATP in this reaction, and added cocarboxylase appeared rapidly as a labile P of the mitochondrial system. In this respect, it must be mentioned that Lipmann (*15*) indicated in his recent experiment on the incorporation of inorganic P^{32} into ATP, that 'gel P' appeared not to be an intermediate in its energy-rich phosphate bond generation.

The data presented here may explain our former results (*5*, *6*), in which the rate of the oxidative phosphorylation of thiamine-deficient rats was lower as compared with that of the normal rats, and the thiamine-deficient rats showed significant decrease in the turnover rate of the labile P of ATP and ADP as compared with normal and pair-fed control rats after the intraperitoneal injection of inorganic P^{32}.

In this respect, Lehninger's report (*21*) attracts our attention. In this paper he reported that the added cocarboxylase elevates the P:O ratio in the presence of pyruvate as a substrate.

SUMMARY

1. Rapid incorporation of P^{32}-cocarboxylase into the terminal P of ATP was observed in the tissue homogenate or especially in the liver cyclophorase system in the presence of α-ketoglutarate or pyruvate.

2. From the experiment using the liver cyclophorase system it was especially indicated that there might be a direct route (not via inorganic P) of phosphate transfer from cocarboxylase to the terminal P of ATP in the presence of α-ketoglutarate or pyruvate.

3. A mechanism assuming that a binding form of cocarboxylase (lipothiamide pyrophosphate) might be formed as an intermediate, may be forwarded from the study in which various amounts of cocarboxylase was incubated with cyclophorase system in the presence of α-ketoglutarate.

4. Approximate rate of the incorporation of P^{32} of P^{32}-cocarboxylase into ATP was compared with that of inorganic P^{32} in the presence of NaF which prevented almost completely the release of inorganic P from cocarboxylase.

5. The rate of the incorporation of P^{32}-cocarboxylase into ATP was not inhibited by 2,4-dinitrophenol.

6. The added cocarboxylase appeared as labile P in the liver cyclophorase system.

This work was supported by a Grant in Aid for Fundamental Scientific Research

for the Department of Education. The authors express their gratitude to Prof. Junkichiro Hirade for his helpful suggestions and Prof. Norio Shimazono for his revision of this paper. The authors also wish to thank Dr. Taizo Matsukawa for his helpful suggestions on the preparation of the cocarboxylase.

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The Journal of Biochemistry, Vol. 42, No. 1, 1955

THIAMINE UPTAKE BY YEAST CELLS

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(Received for publication, October 8, 1954)

Allithiamine[1], a new derivative of thiol-type thiamine (*1*, *2*, *3*), was reported to be much more readily absorbed from the intestinal canal than thiamine. From many other reports, allithiamine was concluded to be *per os* better utilizable than thiamine. This strike character appears to be explained partly by its solubility in lipids. Our previous study (*4*) demonstrated that, unlike thiamine, allithiamine readily penetrated into blood cells due to the simple diffusion process. It is, however, well-known that thiamine present in the normal blood is found, for the most part, to be concentrated within the blood corpuscles and phosphorylated in the form of cocarboxylase. This fact suggests that thiamine, although extremely fat-insoluble, might possibly be accumulated into blood cells by a particular mechanism such as, so-called " active transport process ". But the amount of thiamine uptaken was so small to be detected by our determination method that the author could not clarify the mechanism. In order to study how the derivatives of thiol type thiamine are uptaken by the cells which are able to accumulate thiamine by the active process[2], the present author selected yeast cells as experimental material instead of blood cells, because yeast was demonstrated by Sperber *et al.* (*6*, *7*) to accumulate thiamine by the process involving fermentation and respiration. The author intended first to carry out the further detailed analysis on the thiamine uptake by yeast cells and the comparative studies on transport mechanisms of thiamine and its derivatives by yeast cells and blood cells. The present paper describes the results obtained.

METHOD AND MATERIALS

All experiments were carried out on the samples of the Kotobukiya's bakers' yeast

1) Allithiamine is named also thiamine allyl disulfide (TAD) and its alkyl homologues are named thiamine alkyl disulfides.

2) A similar active transference of thiamine by a strain of *Staphylococcus aureus* was recently reported by K. M. Citron and R. Knox (*5*).

(*Saccharomyces cervisiae*). Thiamine was fluorimetrically determined by the thiochrome method with cyanogen bromide, using Klett fluorimeter (*8*). Thiamine alkyl disulfides and *S*-acyl thiamines[3] (*9*) were determined by modified methods of the thiochrome method (*11*, *12*). The O_2 uptake and the anaerobic CO_2 evolution were measured by Waruburg apparatus.

Using modified Thunberg tubes, the resting cells were suspended in 50 volumes of a medium containing 0.1 *M* phosphate buffer, pH 5.0 (or 0.1 *M* acetate buffer). Thiamine and glucose or other substrates were added from the side arm at the beginning of the experimental runs at 27° under various gas phases (N_2, O_2 or air). With appropriate intervals 5 ml. of the incubation mixtures was pipetted out and centrifuged at once. The cells were further washed with 0.1 *M* phosphate buffer by centrifuging. Both the combined supernatant solutions and washed cells were analyzed for thiamine, respectively. The amount of thiamine appearing in the cells (Fig. 6, Curve 1), of which the greater part was found to be free thiamine was equal to the amount disappearing from the medium (Fig. 6, Curve 2). It is therefore clearly demonstrated that the thiamine disappeared from the medium was neither destroyed nor converted to any thiochrome reaction negative form during these experimental runs. In most of the subsequent work only the decrease in the thiamine concentration of the incubation medium was determined as showing the thiamine uptake by the cells. In order to ascertain whether the cells are proliferating or not in the course of the incubation, the number of the cells during the shaking experiments were counted by Thomas-Zeiss haematometer and it was observed that no appreciable changes in the population occurred at least for 3 hours.

RESULTS AND DISCUSSION

In the presence of glucose the cells took up thiamine vigorously after the 15–20 minutes lag period under both aerobic and anaerobic conditions (Fig. 1, Curves 2 and 3) whereas no accumulation of thiamine took place unless a source of energy such as glucose was available (Fig. 1. Curve 1).

Then the author studied the effect of pH on the thiamine uptake by the resting yeast cells suspending in the acetate buffer with glucose as substrate for the energy-generating systems. The optimal pH for the thiamine uptake was found to be pH 4.5–5.0.

It was also demonstrated that the uptake was accelerated by the additions of potassium ion (Fig. 2, Curve 3) although it proceeded con-

3) *O*, *S*-diacetyl thiamine is one of *S*-acyl thiamine derivatives recently synthesized by Matsukawa and Kawasaki (*9*) in our Research Laboratory and the author ascertained that it was almost as efficacious as thiamine by the prevention assay of B_1 deficiency in *U. domestica* (*10*).

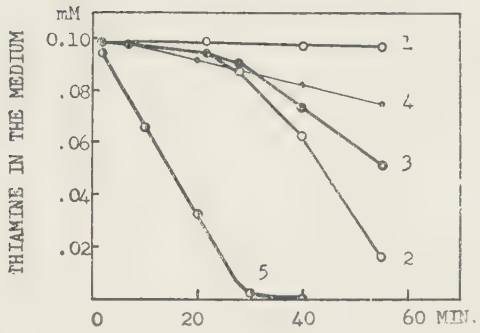

FIG. 1. Thiamine uptake by resting yeast cells under various conditions (*1*).

1. No added substrate, under air. 2. 0.1 *M* glucose, anaerobic (100% N_2) 3. 0.1 *M* glucose, aerobic (100% O_2) 4. 0.1 *M* ethanol, under air. 5. 0.1 *M* glucose, aerobic. The cells were preincubated for 40 min. anaerobically in the presence of 0.1 *M* glucose before thiamine was added.

All experiments were carried out at 27°, pH 5.0 (0.1 *M* phosphate buffer) under shaking.

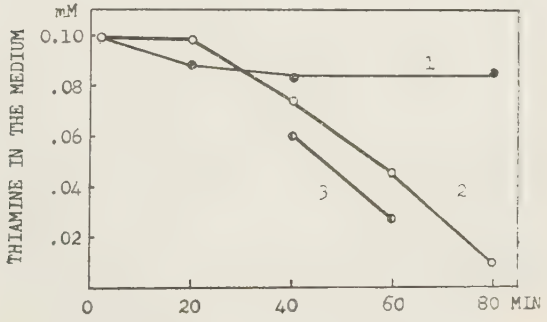

FIG. 2. Thiamine uptake by resting yeast cells (*12*).

1. 0.1 *M* acetate buffer, pH 5.3 2. 0.1 *M* acetate buffer, 0.1 *M* glucose. 3. 0.1 *M* acetate buffer, 0.1 *M* glucose, 0.01 *M* KCl. All experiments were carried out at 27°, pH 5.0 under air.

siderably with no added potassium and phosphate ions (Fig. 2, Curve 2).

Then the influence of the initial thiamine concentrations to the

rates of thiamine uptake was studied in the presence of excess glucose. As shown in Fig. 3, the thiamine concentrations gave no influence to the lag phase and the velocities of thiamine uptake. The fact that the rate of thiamine uptake did not depend upon the concentration gradients of thiamine, demonstrated the process to be a active transport.

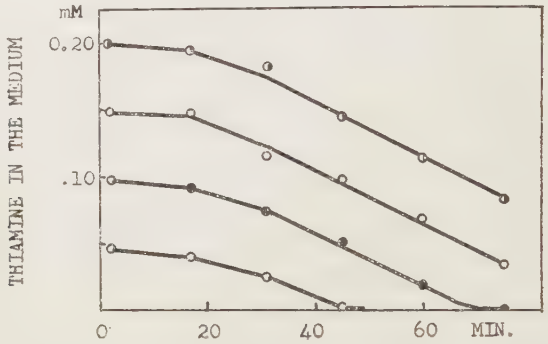

Fig. 3. Relation of thiamine concentration to thiamine uptake by resting yeast cells.

Conditions: 0.1 *M* phosphate buffer, pH 5.0; 0.1 *M* glucose; at 27°, under air.

As shown above, no thiamine uptake was observed in the absence of glucose, so the relation between the amount of glucose added and that of thiamine uptaken was studied. The results showed that the thiamine uptake depends on the glucose concentration in the medium (Fig. 4). In all cases where glucose was present thiamine began to be uptaken after the lag period of about 10–15 minutes irrespective of the glucose concentration. It is noticeable that glucose concentration gave no influence to the length of the lag phase. The lag phase in the thiamine uptake well coincided with that in the respiration or fermentation, during which the energy supply was insufficient to maintain the full rate of the transport. When the fermentation or respiration began to proceed at their maximum velocities (Fig. 5), the vigorous thiamine uptake started and proceeded as long as glucose remained available. These findings were also supported by the fact that the cells preincubated with glucose for 30 minutes uptake thiamine linearly with time as soon as thiamine was added in the incubation medium (Fig. 1, Curve 5).

Using ethanol (Fig. 1, Curve 4) or acetate (Fig. 2, Curve 1) as

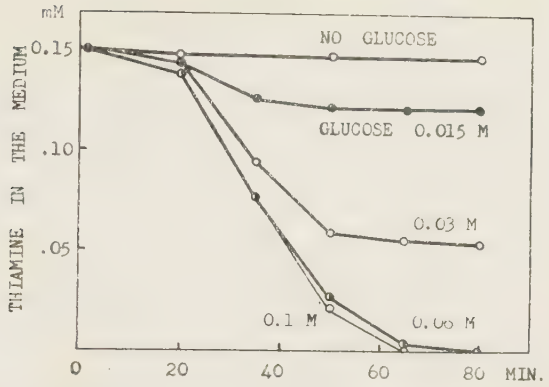

FIG. 4. Effect of glucose concentration on the thiamine uptake by resting yeast cells.

M/15 phosphate buffer, pH 5.0. Under air with shaking at 27°.

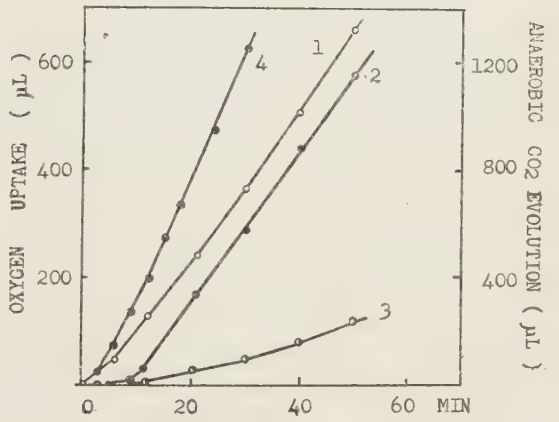

FIG. 5. Oxygen uptake and anaerobic CO_2 evolution by yeast cells. Oxygen uptake (with 10 mg. wet wt. cells): 1. ethanol, 2. glucose, 3. acetate, 4. glucose.

Anaerbic CO_2 evolution (with 10 mg. wet wt. cells).

The final concentration of substrates, 0.02 *M*. Temp., 30°, pH 5.5.

respiratory substrate instead of glucose, thiamine was uptaken aerobically,

although much less than with glucose. There was found the parallelism between the amount of thiamine uptaken and the rate of O_2 uptake (Fig. 5). From these results it was also indicated that the thiamine uptake have close correlation with the exergonic metabolism. It was therefore anticipated that thiamine uptake should be greatly inhibited by the ordinary metabolic inhibitors. The results showed that 0.05 *M* NaF (Fig. 6), 0.01 *M* iodoacetate (IAA) (Fig. 7) and 0.01 *M* NaN_3 (Fig. 8)

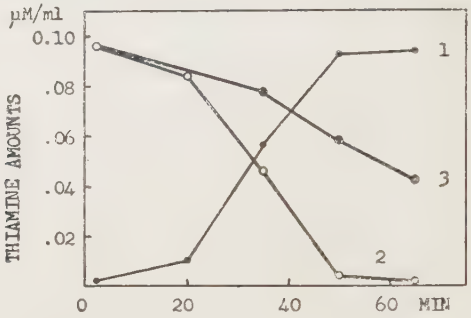

Fig. 6. Effect of NaF on thiamine uptake.

The amounts of thiamine found in the cells (Curve 1) and the media (Curves 2 and 3) contained in 1 ml. of the reaction mixtures were illustrated. All experiments were carried out at 27°, under air in the reaction mixture containing 0.1 *M* glucose, 0.03 *M* phosphate buffer, pH 5.7. In case of Curve 3, 0.05 *M* NaF was further added.

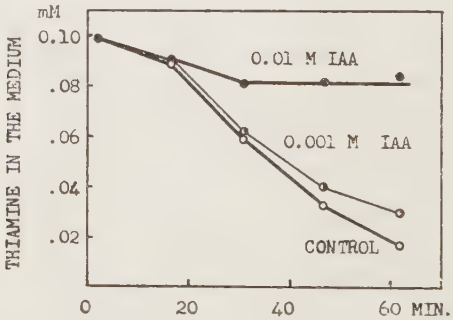

Fig. 7. Effect of iodoacetate on thiamine uptake.

0.1 *M* glucose, 0.1 *M* phosphate buffer, pH 5.7, at 27°, under air.

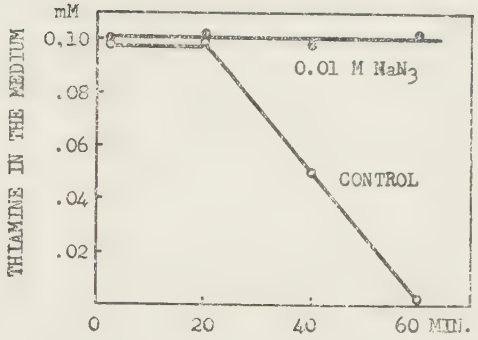

FIG. 8. Effect of NaN_3 on thiamine uptake under anaerobic conditions.

Another conditions same as those in Fig. 7.

greatly inhibited the thiamine uptake under both aerobic and anaerobic conditions with glucose as substrate. Concentrations of IAA and NaF exerting the inhibition of respiration or fermentation are approximately the same as those producing complete inhibition of the thiamine accumulation. This is, however, different in the case of NaN_3. 0.01 *M* NaN_3 exerted complete inhibition of O_2 uptake but inhibited the anaerobic CO_2 evolution only by 20 per cent. The fact that 0.01 *M* NaN_3 inhibited the accumulation of thiamine without inhibiting the fermentation reaction, suggested the coupling of the energy-transferring reactions with the thiamine uptake.

In addition to NaN_3, 2,4-dinitrophenol (DNP) sharply inhibited the thiamine uptake in the concentration giving no appreciable influence to the O_2-uptake (Fig. 9). 0.005 *M* arsenate also greatly inhibited the thiamine uptake by the yeast suspended in acetate buffer devoid of phosphate ion, and the inhibition was fairly reversed by the addition of 0.05 *M* phosphate (Fig. 10). These facts demonstrated that the phosphate cycle took place in the process. It was anticipated from these results that the cells might be able to uptake thiamine by the addition of ATP without glucose. It was, however, found that thiamine was not uptaken at all even in the presence of 0.2 *M* ATP.

After the yeast cells were shaken in the medium containing glucose and 0.1 *M* phosphate buffer for 1 hour at 26° under N_2 gas bubbling, the cells were centrifuged and the resulting supernatant solution was discarded. After washing twice by the addition of 0.1 *M* phosphate buffer

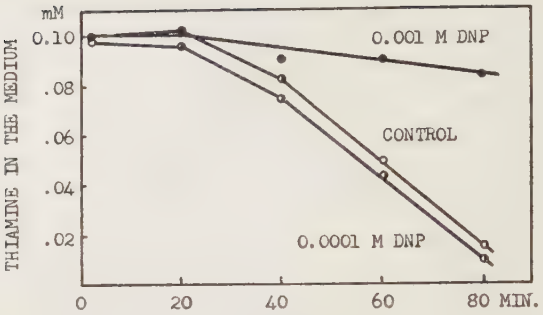

Fig. 9. Effect of 2, 4-dinitrophenol on thiamine uptake.
Conditions same as those in Fig. 7.

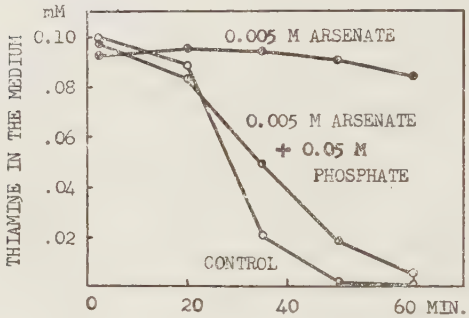

Fig. 10. Effect of arsenate on thiamine uptake and the reversal of arsenate inhibition by phosphate.
Conditions same as those in Fig. 7, except that 0.1 *M* acetate buffer was used instead of phosphate.

and centrifuging, the cells were resuspended in phosphate buffer. The pre-incubated cells thus obtained acquired the ability to accumulate thiamine even in the absence of glucose (Fig. 11–a). The thiamine uptake by the preincubated cells was also inhibited by 0.01 *M* NaN_3 (Fig. 11–b), 0.01 *M* IAA (Fig. 11–c) or 0.005 *M* arsenate (using acetate buffer in place of phosphate buffer) (Fig. 11–d). The presence of glucose in the preincubation medium was indispensable to enabling the cells to uptake thiamine and the addition of phosphate increased the effect (Fig. 11–a), while the cells preincubated with 0.01 *M* arsenate has no ability to uptake thiamine (Fig. 11–d).

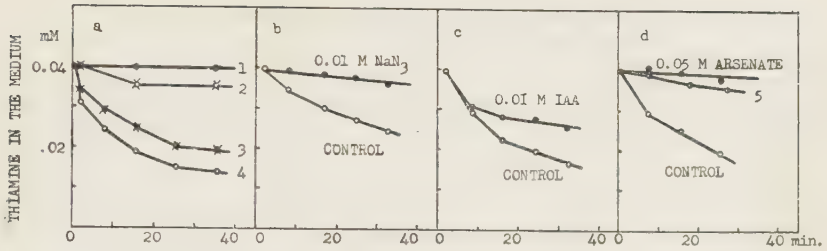

FIG. 11. Thiamine uptake by preincubated yeast cells with glucose.

Expt. a. Conditions for preincubation: Non-preincubated (Curve 1). Preincubated in water (Curve 2), in 0.5 *M* glucose (Curve 3) and in 0.5 *M* glucose plus 0.1 *M* phosphate buffer (Curve 4) for 40 min. at 27° under air. pH 5.7.

Conditions for thiamine uptake: All experiments were carried out in the medium containing 0.1 *M* phosphate buffer, pH 5.7 and no added glucose at 27° under air.

Expts. b, c, and d. Effects of NaN_3, iodoacetate, arsenate on thiamine uptake by the preincubated cells.

Conditions for preincubation: Same as those in Curve 4. But in Curve 5, the cells were preincubated in the medium containing 0.2 *M* glucose and 0.05 *M* arsenate with 0.1 *M* acetate buffer instead of phosphate buffer.

Conditions for thiamine uptake: 0.1 *M* phosphate buffer, pH 5.7 at 27° under air.

The conditions for the preincubation were quite the same as those used by Schmidt, Hecht and Tannhauser (*13*) in their studies on the phosphate uptake resulting in the accumulation of metaphosphate in the cells. Under the conditions the yeast assimilated not only orthophosphate but also glucose so that polysaccharides should have been accumulated in the cells. In fact, the Q_{O_2} values were found under author's experimental conditions to be 8.0 and 26.1 before and after the preincubation, respectively. Accordingly, at least the following two possibilities seemed to bear explaining the effect of the preincubation. Firstly, the metaphosphate accumulated during the preincubation might serve as phosphagenics for the process, and secondly, the polysaccharides formed during the preincubation might serve as the endogenous substrates for the energy-generating process.

The author studied whether some thiamine derivatives might be

uptaken by the yeast just like as thiamine. The derivatives used contain thiamine propyl disulfide (TPD), thiamine disulfide and *O*,*S*-diacetyl thiamine (DAT). The results showed that either TPD (Fig. 12), thiamine disulfide or DAT (Fig. 13) was hardly uptaken by the cells. Accordingly, unlike thiamine, these derivatives of the thiol type thiamine could not be uptaken unless they were converted to thiamine by reduction or hydrolysis. From the anticipation that hydroxylthiamine would inhibit the thiamine uptake competitively, the effect of hydroxylthiamine on thiamine uptake was investigated. But the result showed that hydroxylthiamine exerted nearly no influence, although it somewhat elongated the lag phase.

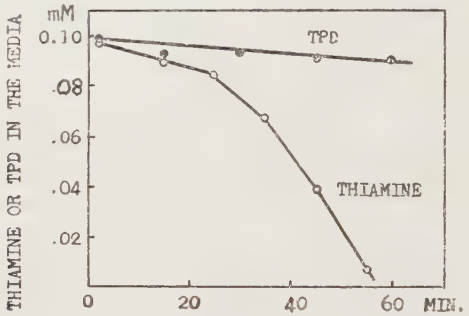

Fig. 12. Thiamine propyl disulfide (TPD) uptake by resting yeast cells.

Conditions same as those of the Control in Fig. 7.

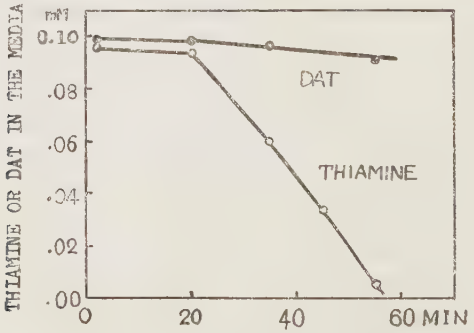

Fig. 13. *O*, *S*-diacetyl thiamine (DAT) uptake by resting yeast cells.

Conditions same as those of the Control in Fig. 7.

When the resuspended cells once having accumulated thiamine in the high concentration by the above-mentioned process were shaken in phosphate buffer, a little of thiamine slowly reappeared in the medium (Fig. 14). However, the addition of glucose fairly protected this leakage. Accordingly, the maintenance of thiamine once accumulated in the cell seemed to require the active metabolic state of cells.

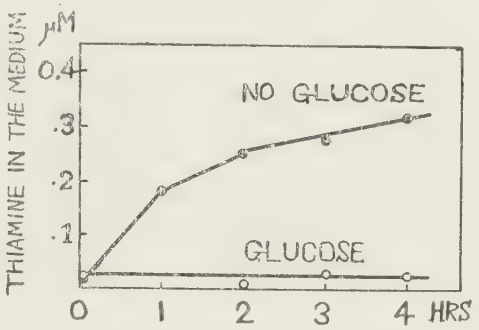

FIG. 14. Maintenance of thiamine once uptaken by resting yeast cells.

After uptaking thiamine for 1 hour under the same conditions as those of Case 2 in Fig. 1, washed cells were resuspended in 0.1 *M* phosphate buffer, pH 5.0 in the presence or absence of 0.1 *M* glucose.

From all the above-mentioned results the author concluded that thiamine may be accumulated into yeast cells by a typical active transport mechanism. However, the present paper provided only phenomenal findings that thiamine uptake by yeast had a close correlation to carbohydrate and phosphate metabolisms, giving nothing to clarify the mechanism in the enzymic level by which thiamine is transported across cell wall and pumped up within the cells. In order to ascertain whether thiamine would be trapped on the cell membrane by a surface enzyme, for example, thiaminokinase, further enzymic and histochemical studies should be performed.

It is noticeable that yeast did not uptake thiol-type thiamines which were demonstrated to penetrate into blood cells according to the process of solution in, and diffusion through, the lipid membrane (*4*), Previously, the author demonstrated that the thiamine alkyl disulfides by reduction (*4*) and *S*-acyl thiamines by hydrolysis (*14*) were readily converted

to thiamine in vivo. Therefore, a possible interpretation may be put for the better absorbabilities of thiol-type thiamines that they would be absorbed from intestinal canal by simple diffusion due to their lipid solubility as well as by the normal mechanism of thiamine absorption because of their previous conversion to thiamine.

SUMMARY

In the presence of glucose baker's yeast uptook thiamine after 15 minutes lag period both under aerobic and anaerobic conditions. The preincubation with glucose enabled the cells to uptake thiamine without lag phase even in the absence of glucose. The effects of several metabolic substrates or inhibitors were investigated under various conditions. It was concluded that yeast uptook thiamine according to a typical active transport involving the energy-generating and -transferring systems. Thiol-type derivatives of thiamine were not uptaken under the same conditions.

RESUMO

Ĉeeste de glukozo bakgisto enprenis tiaminon post 15 minutoj da induktperiodo sub ambaŭ aeroba kaj neaeroba kondiĉoj. La ĉeloj kiuj estis antaŭe kovitaj kun glukozo enprenis tiaminon sen indukto eĉ en neesto de glukozo. Influoj de pH, glukozo-, thiamino- densecoj kaj malhelpantoj sur tiamino-enpreno estas esploritaj sub diversaj kondiĉoj por analizi rilaton al karbohidrato- kaj fosfato-metaboloj. Konklude estis klarigite ke gisto enprenis tiaminon laŭ aktiva transporto-mekanismo kunligita kun energio-generanta kaj -transdonanta sistemoj kaj ke tiol-tipaj derivaĵoj de tiamino estis ne enprenitaj sub sama kondiĉo.

The author is deeply indebted to Miss Y. Kinosita for technical assistance. The author's thanks are also due to Dr. S. Matsumiya of Kotobukiya Co., Ltd. for generous gifts of fresh baker's yeast and to Dr. T. Matsukawa for the supply of all samples of thiamine derivatives. This work was supported in part by a grant from the Ministry of Education.

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The Journal of Biochemistry. Vol. 42, No. 1, 1955

BIOCHEMICAL STUDIES ON SULFATE-REDUCING BACTERIA

IV. REDUCTION OF THIOSULFATE BY CELL-FREE EXTRACT

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(Received for publication, October 12, 1954)

Reduction of thiosulfate by sulfate-reducing bacteria or by other bacteria has been observed since several decades (*1*). On sulfate reducing bacteria, Baars studied reduction of thiosulfate as well as of sulfate in 1930 (*2*) and Stephenson and Stickland found thiosulfate-reduction with hydrogen gas in 1931 (*3*), and recently Postgate reported that 4 moles of molecular hydrogen was absorbed in the reduction of each 1 mole of thiosulfate (*4*). On the other hand, release of hydrogen sulfide by other bacteria was found by Sasaki and Otsuka (*5*) and studied by Tarr on *Proteus vulgaris* (*6*). Recently, Mitsuhashi and Matsuo reported that in cell suspension as well as in cell-free extract of *Proteus vulgaris*, cultivated on agar containing thiosulfate, hydrogen sulfide and sulfite were formed from thiosulfate in the presence of suitable hydrogen donors such as formate (*7*). They found that the reaction requires other factor besides formic dehydrogenase. The mechanism of the thiosulfate reduction by these organism, however, has not yet been elucidated.

The authors studied the reduction of thiosulfate as well as that of sulfate and sulfite by sulfate-reducing bacteria and proposed the following Scheme(1) for the reaction (*8*, *9*, *10*).

$$\underset{\text{hydrogenase}}{H_2} \cdots\cdots \text{intermediary carrier} \cdots\cdots \underset{\text{reductase}}{S_2O_3^{--}} \qquad (1)$$

In the present paper, it is reported that the cell-free extract of the sulfate-reducing bacteria is able to catalyse the reduction of thiosulfate to sulfite and sulfide with molecular hydrogen.

MATERIALS AND METHODS

Preparation of Cell-free Extracts—The strain and the conditions of cultivation were

the same as used in the previous reports: (*10*). After being harvested, washed and preincubated in hydrogen atmosphere, cell paste of the sulfate-reducing bacteria was ground with quartz sand or alumina for 10 minutes and then extracted with *M*/15 phosphate buffer at 7.0. After centrifuging the extract at 4,000 r.p.m. for 50 minutes, slightly turbid, yellow supernatant was obtained, which was used for experiments. It had activities of hydrogenase and lactic, pyruvic and formic dehydrogenase.

Preparation of Sulfur Compounds—Colloidal sulfur solution (*11*), sodium dithionate (*12*), sodium trithionate, sodium tetrathionate and sodium pentathionate (*13*) were prepared. None of these contained thiosulfate at any trace, since they could not reduce iodine at all.

Measurements of Hydrogen Absorption—The activity of thiosulfate reduction was determined usually by measuring the rate of hydrogen absorption in Warburg manometer. The conditions were as follows. The main compartments of the vessels received the cell-free extract 2 ml. and water 0.4 ml., and the side arms 0.05 *M* $Na_2S_2O_3$ 0.2 ml. and methyl viologen (1:2,500) 0.3 ml., and the center wells 10 per cent potassium hydroxide 0.2 ml. The gas phase was pure hydrogen and temperature was 30°.

For the measurement of hydrogenase activity, the conditions were the same as those described above, but the content of the side arm was replaced by 0.002 *M* methylene blue 0.5 ml.

Determination of Sulfite and Hydrogen Sulfide—Determination of sulfite was carried out for reaction mixtures in the main compartments of the Warburg vessels. The procedure was as follows. Two vessels were used for the reaction. After the reaction, immediately 0.5 ml. of 30 per cent formaldehyde was added to the reaction mixture in one vessel and water in the other vessel. Each mixture was acidified with several drops of 2 *M* HCl, and thereafter 5 ml. of *N*/100 iodine solution was added. Then the contents were transfered to 50 ml. Erlenmeyer flask and titrated with *N*/100 $Na_2S_2O_3$ in the presence of starch as an indicator. The difference of the titration values obtained indicates the amount of sulfite, formed during the reaction, since sulfite combines with formaldehyde and becomes unable to reduce iodine. Preliminarily, in order to test the accuracy of this method, titration was carried out for the extracts, to which definite amounts of sulfite and thiosulfate were added. The obtained values were found to agree well with the additive values.

For the determination of hydrogen sulfide, produced in the manometric experiments, the sulfide content of alkali in the center well of the vessel was measured as follows: water, made free from air by boiling, was added to the center well and the content was all pipetted and poured into 5 ml. of zinc acetate solution acidified with acetic acid. After the solution was diluted to 6 ml., sulfide was determined colorimetrically by the method of St. Lorant (*14*).

This method for measuring sulfite may give low values, because evolution of hydrogen sulfide from the neutral reaction mixture in the main compartment is not sufficiently complete, and sulfide is very autoxidizable.

In some experiments, hydrogen sulfide developed from thiosulfate was measured. Reaction was carried out with bubbling hydrogen and hydrogen sulfide formed was

driven off from the reaction mixture and estimated by St. Lorant's method.

Reduction of Thiosulfate with Reduced Methyl Viologen—As methyl viologen is colorless in the oxidized form and blue in the reduced, oxidation of reduced methyl viologen is followed by the fading of the blue color of the reduced form. This method for measurements of thiosulfate reduction depends on this fact.

The reaction was carried out in Thunberg tubes. The cell-free extract, which has been dialysed at 4° overnight against *M*/75 phosphate buffer, was placed in the tubes and 1 ml. of thiosulfate solution or others was put in the hollow stoppers. After the tubes were evacuated, the reduced methyl viologen in phosphate buffer, which was reduced by zinc sands and filtrated with a glass filter to make free from zinc, was poured into the tubes anaerobically. The tubes were incubated for 10 minutes and then the contents of the hollow stoppers were poured into the tubes and the time required for complete decolorization of the blue reduced methyl viologen was measured at 30°.

Preparation of Cell Suspension of E. Coli and Its Cell-free Extract—In order to obtain cell suspension with hydrogenase activity, *E. coli* was grown in deep stationary culture at 37° for 12 hours in a medium containing 5 per cent glucose, 1 per cent peptone and 1 per cent meat extract. The cells were collected by centrifuging, washed and suspended in *M*/15 phosphate buffer, pH 7.0. Cell-free extract was prepared by grinding the cell paste with alumina and extracting it with phosphate buffer. The extract had also hydrogenase activity.

EXPERIMENTS AND RESULTS

Thiosulfate Reduction by Cell-free Extracts of the Sulfate-reducing Bacteria—The cell-free extract from the ground cells with alumina did not show hydrogen uptake in the presence of sulfate or sulfite in spite of its strong hydrogenase activity, and only the slow reduction of thiosulfate with gaseous hydrogen was observed. But in the presence of a very small amount of methyl viologen, hydrogen uptake due to the thiosulfate reduction was very much accerelated. The reduction of thiosulfate was always preceeded by the reduction of the dye, which was indicated by appearance of blue color of the reduced form of methyl viologen. Therefore, it may be that methyl viologen functions as a hydrogen carrier and this reaction takes place possibly following the Scheme(2).

$$\underset{\text{hydrogenase}}{H_2 \xrightarrow{\uparrow}} \text{.....methyl viologen.....} S_2O_3^{--} \qquad (2)$$

On the other hand, even in the presence of methyl viologen, sulfate was not reduced with hydrogen. In the case of sulfite slow hydrogen uptake occurred. The results were shown in Figs. 1 and 2, in which hydrogen uptake were plotted against time.

As can be seen from Fig. 1, the amount of absorbed hydrogen was

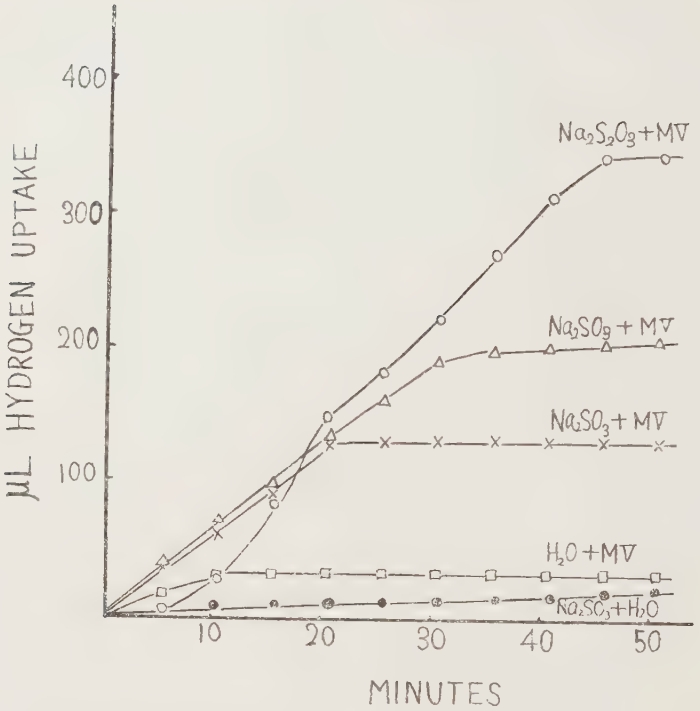

Fig. 1. Hydrogen uptake in thiosulfate reduction.

Conditions; Manometers were employed. The main compartments received cell-free extract (0.25 mg. N/ml.) 2.0 ml., the side arms methyl viologen (1:2,500) 0.2 ml. and *M*/50 $Na_2S_2O_3$ 0.2 ml. (○) or *M*/25 (△) or *M*/50 (×) Na_2SO_3 0.2 ml., the center wells 20% KOH 0.2 ml. Atmosphere: hydrogen. Temperature: 30°.

approximately 4 moles for a mole of thiosulfate and 3 moles for a mole of sulfite. These results indicated that thiosulfate and sulfite were reduced to hydrogen sulfide completely.

From these figures, especially from Fig. 2, it was also observed that in thiosulfate reduction the rate of hydrogen uptake was considerably large at first, and thereafter it dropped to the level of that in sulfite reduction. As the bending of the curve was situated a little over 10 μM hydrogen consumption, which was equivalent to the amount of the added thiosulfate, it appears that the following two reactions occurred successively.

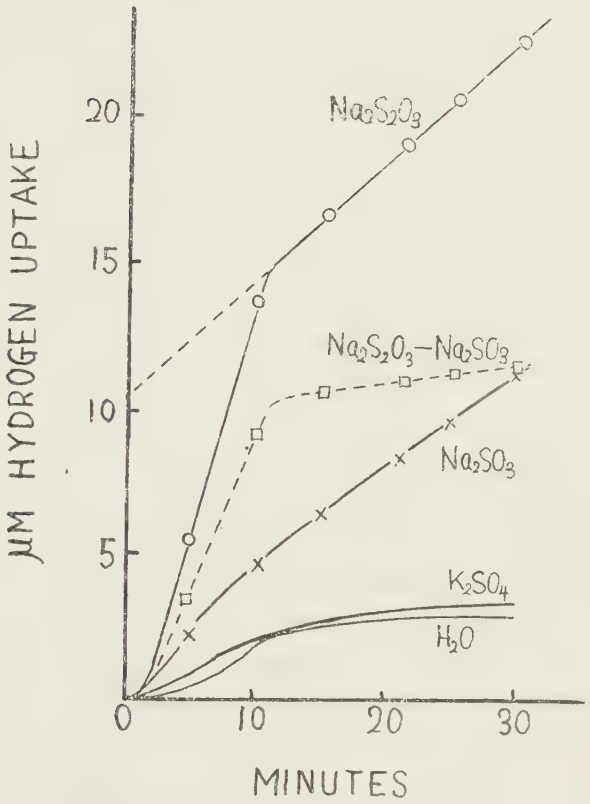

FIG. 2. Hydrogen uptake in thiosulfate reduction. Conditions are indicated in " Methods ".

$$H_2S_2O_3 + H_2 = H_2S + H_2SO_3 \quad (3)$$
$$H_2SO_3 + 3H_2 = H_2S + 3H_2O \quad (4)$$

Hydrogen uptake in the first rapid stage may be due to the Reaction (3), as well as to the Reaction (4), which takes place slowly at the consumption of the sulfite formed by the Reaction (3). The difference of the curve of thiosulfate and that of sulfite, expressed in broken line in Fig. 2, could be considered to indicate the hydrogen uptake owing to the Reaction (3) alone. The hydrogen uptake, indicated by this curve, almost stopped at 230 μl. *viz.* 10 μM, which was equal to the moles of the added thiosulfate. Then we tried to prove the intermediary forma-

tion of sulfite in the course of the reaction. As soon as the rapid hydrogen uptake in the first stage dropped, the Warburg vessels were disconnected from the manometers and the sulfite content of the reaction mixtures in the main compartments and the sulfide content of the alkali in the center wells were determined. The results are indicated in Table I.

TABLE I

The Formation of Sulfite and Sulfide by the Reduction of Thiosulfate

Exp. No.	Product measured	Amount formed	Control	Hydrogen absorbed*
1	SO_3^{--}	9.1 μM	2.5 μM	12.7 μM
2	"	6.7	2.0	14.8
3	H_2S	4.5	0.2	16.7
4	"	5.2	0.1	—

The methods for measurements are indicated in "Methods". Added thiosulfate: 15 μM, Temperature: 30°.

* The values of hydrogen absorption were obtained by extraploation of the last part of hydrogen uptake curves to time zero. (As indicated in Fig. 2 by the dotted line)

It was ascertained that sulfide and sulfite were produced in the reduction of thiosulfate. The amounts of them, however, were not so much as expected from the Eq. (3). It is perhaps due to the autoxidation of both and consumption of sulfite by the succeeding reduction of Reaction (4) as well as to the incomplete absorption of hydrogen sulfide in the alkali in the center wells.

The two stages of the thiosulfate reduction was also observed in the experiment of hydrogen bubbling. The formed hydrogen sulfide was at first very fast and then dropped to the level of the sulfite reduction as indicated in Fig. 3, in which the effect of methyl viologen is also shown. These results agree well with those of the hydrogen absorption.

Reduction of Colloidal Sulfur and Polythionates—When thiosulfate was replaced by colloidal sulfur or polythionates in the experiments with manometer, it was found that sulfur and tetrathionate were reduced well. As 338 μl, *e.g.* 15 μM, of gaseous hydrogen was absorbed rapidly for the reduction of 5 μM tetrathionate. Trithionate was reduced only very slowly. It was not ascertained that trithionate itself was reduced directly or the products of its decomposition were reduced. Dithionate, penta-

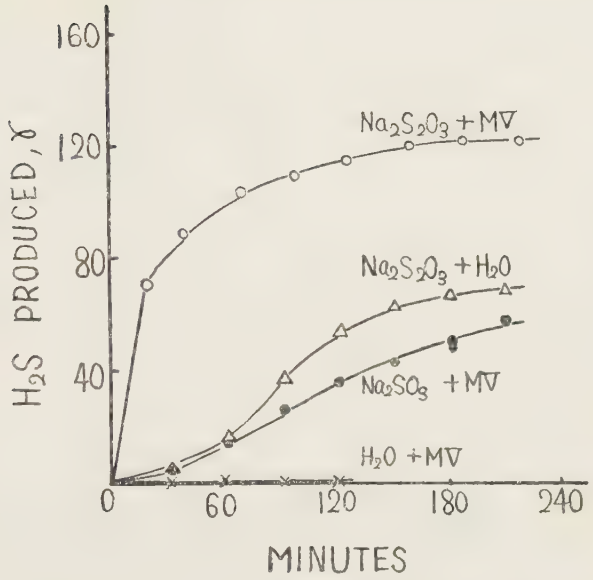

Fig. 3. Hydrogen sulfide formation in thiosulfate reduction. Conditions; The reaction mixture contained 3 ml. of the cell-free extract (1.4 mg. N), 0.2 ml. of methyl viologen 1:2,500, 2.21 μM $a_2S_2O_3$ or 10 μM Na_2SO_3. Total volume: 3.7 ml. The reaction mixture was bubbled by molecular hydrogen and formed hydrogen sulfide was measured. Temperature: 30°.

thionate and cystine were not reduced.

Availability of Dyes for the Intermediary Carrier—Besides methyl viologen, other dyes with different redox potentials were tested. They were benzyl viologen, neutral red, safranine, janus green, methylene blue and malachite green. When they were added instead of methyl viologen, hydrogen absorption, indicating thiosulfate reduction, scarcely took place except in the case of benzyl viologen, although they were converted to the reduced forms. In the case of benzyl viologen, hydrogen uptake occurred slowly (Fig. 4).

In the case of the reduction of colloidal sulfur, hydrogen was also absorbed, when safranine was used as an intermediary carrier. The reaction rate was about half of that for methyl viologen.

When the concentration of methyl viologen was increased, the reaction rate of thiosulfate reduction was correspondingly increased. As

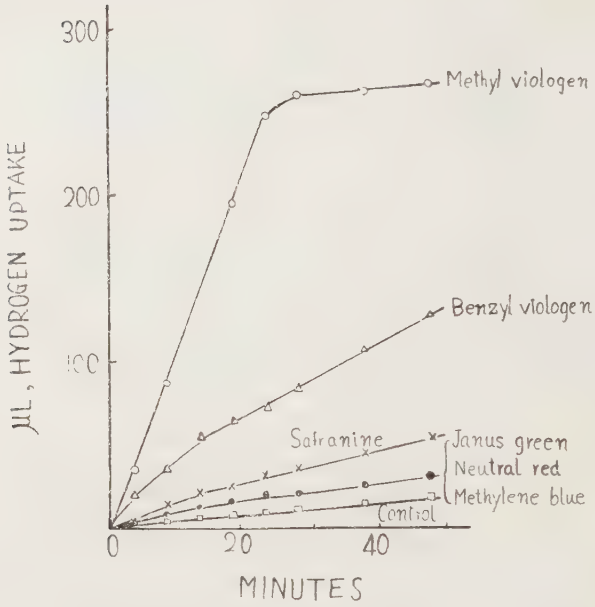

FIG. 4. Thiosulfate reduction with hydrogen in the presence of various dyes.

Conditions: Manometers were employed. Main compartments received cell-free extract 2 ml., side arms dye solution 0.3 ml. (1:2,500, but 1:5,000 in the case of benzyl viologen) and 0.05 *M* $Na_2S_2O_3$, 0.2 ml. and center wells 20% alkali, 0.2 ml. Atmosphere: hydrogen. Temperature: 30°.

an example, the amount of hydrogen absorbed for an hour was 664 μl. when the concentration of methyl viologen was 1.1×10^{-5} *M*, and 819 μl., when 8×10^{-4} *M*.

Influence of pH—The hydrogen uptake was determined at different pH. The reduction took place only on range of pH 7.0–7.5. The velocity of hydrogen uptake was larger at pH 7.5 than at pH 7.0. But as the values of the reaction rate were fluctuated at pH 7.5, experiments were carried out usually at pH 7.0.

The hydrogenase activity of the extracts measured from hydrogen absorption with methylene blue had maximum also near pH 7.5 (Fig. 5).

Influence of Concentration of the Extract—The experiments were carried out with the twice, fourth and eighth diluted extract. The original

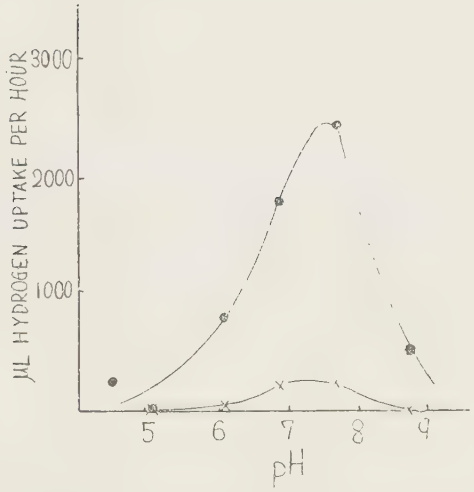

FIG. 5. Influence of pH on hydrogen absorption.

Acceptor, o: Methylene blue, ×: $Na_2S_2O_3$, supplemented with a small amount of methyl viologen. Phosphate and acetate buffer were employed.

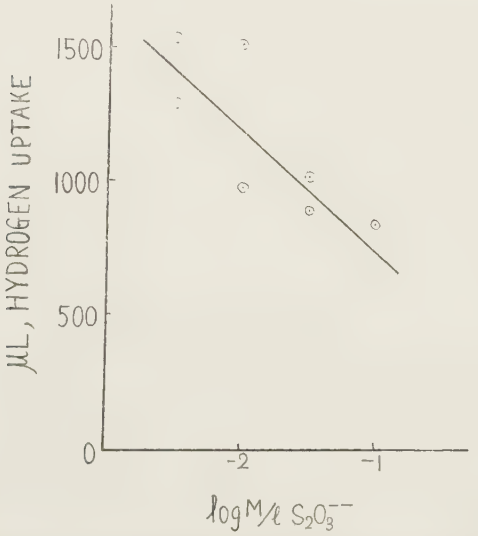

FIG. 6. Influence of thiosulfate concentration on hydrogen absorption.

extract contained 0.70 mg. nitrogen per ml. The rates of hydrogen uptake were found to be proportional with the extract concentration.

Influence of Thiosulfate Concentration—The reaction rates at different concentration of thiosulfate are indicated in Fig. 6. The optimum concentration of thiosulfate lies at less than 10^{-2} *M*.

Thiosulfate Reduction with Reduced Methyl Viologen—Thiosulfate and methyl viologen, reduced with zinc metal, were mixed in vacuum in Thunberg tubes in various conditions and the time required for complete fading of the blue color were measured.

In the absence of the extract, the reduction of thiosulfate did not take place at all. The results in the presence of the dialyzed extract are listed in Table II.

Table II

Thiosulfate Reduction with Reduced Methyl Viologen

In the Thunberg tube	In the hollow stopper	Time for decolorization
Extract 2 ml.+ $8\times10^{-4}M$ methyl viologen 15 ml.	0.05 *M* $Na_2S_2O_3$, 2 ml.	28 *min.*
Water 2 ml.+ "	Water 2 ml.	∞*
Boiled extract 2 ml.+ "	0.05 *M* $Na_2S_2O_3$, 2 ml.	∞*

* The blue color of reduced methyl viologen remained completely after 24 hours incubation.

It is suggested from the results that thiosulfate reduction with reduced methyl viologen is catalysed by a non-dialyzable and thermolabile substance in the extract, probably an enzyme.

Colloidal sulfur and tetrathionate, on the other hand, were reduced by reduced methyl viologen instantly even in the absence of the extract, so the reactions seem spontaneous.

Reduction of Thiosulfate by Other Bacteria—As E. coli is known to possess hydrogenase activity (*16*), experiments were performed on the reduction of thiosulfate using the cell suspension and the cell-free extract. Although the reduction of dyes, including methyl viologen, took place in both cases, the hydrogen uptake due to the thiosulfate was not observed. In the cells of *E. coli*, there may be none or very little amount of the enzyme for thiosulfate reduction.

Hydrogenase preparation from Azotobacter, gifted kindly by Dr.

Y. Oda, did not reduced methyl viologen at all as well as thiosulfate.

DISCUSSION

In the previous papers (*8*, *9*, *10*), we suggested the presence of specific reductases to sulfate, sulfite and thiosulfate in the cells of sulfate-reducing bacteria mainly from the results of inhibitory experiments. In the cell-free extracts of the bacteria, although activity of sulfate reduction was not observed, thiosulfate reduction was found to take place. The fact that the reduction of thiosulfate with reduced methyl viologen was catalyzed by the extract, indicates the presence of an enzyme, specific to thiosulfate reduction. The stimulating effect of methyl viologen on the reduction of thiosulfate with hydrogen in the cell-free extract may be due to its action as an intermediary carrier. It was reduced with hydrogen by hydrogenase and then oxidized with thiosulfate by the enzyme in the extract of the sulfate reducing bacteria. The extract of *E. coli*, as well as its living cell suspension, reduces methyl viologen with hydrogen, but did not reduce thiosulfate with the reduced type of the dye, perhaps owing to the absence of the enzyme for the thiosulfate reduction.

For the activity of this enzyme for thiosulfate reduction two mechanisms are considered. In one case, the enzyme may directly ctivate thiosulfate for reduction, that is, it may be a reductase. In the other case, it may accerelate the decomposition of thiosulfate to sulfite and elemental sulfur, which is to be reduced spontaneously by reduced methyl viologen, as follows.

$$S_2O_3^{--} = S + SO_3^{--} \quad (4a)$$

$$S + 2H \text{ (reduced methyl viologen)} = H_2S \quad (4b)$$

The latter was denied, because the reduction of thiosulfate proceeded scarcely in presence of safranine in place of methyl viologen. If the latter case were correct, the reaction should have taken place as quickly as in the presence of methyl viologen, as the elemental sulfur is reduced in presence of safranine as quickly as in presence of methyl viologen. In this case safranine itself has no inhibitory effect on thiosulfate reduction, as the addition of safranine does not cause any depression of the rate of thiosulfate reduction in the presence of methyl viologen. Therefore, the enzyme activating thiosulfate for the reduction may be a reductase.

The free energy requirement for the reduction of thiosulfate (ΔF),

$$S_2O_3^{--} + H_2 = H_2S + SO_3^{--}$$

is 140 cal. (pH 7.0) and rH_0 for the reaction is -0.1, and this value in-

creases as the concentration of the reactants are decreased.

The rH_0 of the dyes used in the experiments of the intermediary carriers were as follows; methyl viologen: —0.9, benzyl viologen: 2.0, neutral red: 2.7, janus green: 5.6, safranine: 5.6, methylene blue: 14.4 (*17*). That only methyl viologen and benzyl viologen, the rH_0 values of which are the lowest, are available as the intermediates, is quite reasonable. This ability of the viologen dyes depends on their low redox potentials as well as on their suitable structures. On the other hand, ΔF for the reduction of sulfur, $S+H_2=H_2S$ at pH 7.0, is —6,980 cal. and rH_0, 5.1. It is quite probable that the colloidal sulfur are reduced with hydrogen gas also in presence of safranine as well as of methyl viologen.

An attempt to show the intermediary formation of sulfite during thiosulfate reduction to sulfide in the living cell suspension of the sulfate-reducing bacteria, did not succeeded. In the living cell suspension, contrary to the extract, sulfite reduction takes place several times more quickly than that of thiosulfate, so the negative results for sulfite does not deny the intermediary formation of sulfite in the reduction of thiosulfate. The thiosulfate reductase, found in the cell-free extract, may take part in the thiosulfate reduction in the living cells.

From the experiments concerning the reduction of tetrathionate, it was confirmed that tetrathionate is reduced spontaneously with reduced methyl viologen, and 3 moles of hydrogen are consumed for reduction of 1 mole of tetrathionate in presence of the extract of the sulfate-reducing bacteria and methyl viologen. It appears that the products of the latter reaction are sulfite and sulfide, which are produced after the intermediate formation of thiosulfate. The reaction may be as following.

$$S_4O_6^{--} \xrightarrow{(a)\ 2H} 2\,S_2O_3^{--} \xrightarrow[\text{thiosulfate reductase}]{(b)\ 4H} 2\,SO_3^{--} + 2\,H_2S \quad \text{(a)}$$

The reaction phase of the reduction of tetrathionate to thiosulfate may take place without a specific enzyme in presence of methyl viologen, so the presence of so-called " tetrathionase ", being considered to catalyze specifically the Reaction (a) (*15*), must be examined enzymologically.

The intermediary hydrogen carrier acting in living cells was not elucidated. The fact, that in the cell-free extract, without addition of methyl viologen, thiosulfate reduction took place, though slowly, may indicate the presence of the natural intermediary hydrogen carrier in the extract. Concerning with it, existence of a plentyful amount of a

cytochrome-like pigment in the cells and the extract of sulfate-reducing bacteria is worthy of note (*18*).

SUMMARY

Cell-free extracts from cells of sulfate-reducing bacteria ground with alumina has an ability to reduce thiosulfate to sulfite and sulfide and then to sulfide slowly with an equivalent amount of molecular hydrogen in the presence of methyl viologen as an intermediary carrier. This reaction proceeded in two phases, hydrogenation of the dye by hydrogenase and thiosulfate reduction with the reduced dye catalysed by a specific enzyme. The reduced methyl viologen was found to reduce thiosulfate in presence of the dialysed extract. The conditions of the reactions were investigated.

The authors wish to express their gratitude for kind guidance made by Dr. T. Soda and for the valuable advice rendered by Dr. H. Tamiya and Dr. S. Akabori, and wish to thank Dr. A. Takamiya for giving us methyl viologen and Dr. Y. Oda for gifting hydrogenase preparation of Azotobacter. The expences of the work were aided partly by the Scientific Research funds from the Ministry of Education.

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The Journal of Biochemistry, Vol. 42, No. 1, 1955

STUDIES ON TAKA-AMYLASE A

III. CARBOHYDRATE COMPONENT IN TAKA-AMYLASE A

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(Received for publication, October 14, 1954)

In the previous paper (*1*) which dealt with the amino acid composition of taka-amylase A from "Takadiastase Sankyo", it was reported that the recovery of amino acid residue from the acid hydrolysate of the enzyme protein was about 93 per cent, while N recovery was as high as 99 per cent. Further, purified taka-amylase A gives positive Molisch's reaction, as described by Akabori *et al.* (*2*), and Hanrahan and Caldwell (*3*). These facts provide the evidences for the presence of carbohydrate component in taka-amylase A portein.

Several investigators (*4*, *5*) indicated the presence of carbohydrate groups as integral constituent of crystallized proteins. In such a case, however, the criticism is always necessary on the purity of the material. Hayashi (*6*) has previously reported the presence of carbohydrate in taka-amylase, but the material used in his experiment was not crystalline, therefore, the authors examined very carefully whether carbohydrate component is the integral constituent or contaminated substance in the enzyme protein.

The present communication deals with the study on the carbohydrate component in taka-amylases prepared in crystalline state from "Taka-diastase-Sankyo" and from a synthetic culture media of *Asp. oryzae*.

EXPERIMENTS AND RESULTS

Materials and General Method

Taka-amylase A—Crystalline taka-amylase A obtained from "Taka-diastase Sankyo" by the method of Akabori *et al.* (*2*), was recrystallized three times from aqueous acetone.

Taka-amylase from Synthetic Culture Solution—Crystalline taka-amylase was obtained from a synthetic culture solution of *Aspergillus oryzae* by the method of Akabori *et al.* (*7*).

Determination of Total Carbohydrate—Orcinol-H_2SO_4 method according to Sφren-

sen and Haugaard (*8*) was used for the determination of total carbohydrate. The procedure used in this experiments is as follows: An aliquot of 0.5 ml. of the solution of taka-amylase in water or *N* NaOH was pipetted into the test tube, 0.5 ml. of 2 per cent orcinol in 30 per cent H_2SO_4 and 3.0 ml. of 70 per cent H_2SO_4 were added and heated at 80° for 20 minutes. After cooling 2.0 ml. of 70 per cent H_2SO_4 was added to the solution and the colour density was measured at 530 mμ. All procedures were carried out in the dark.

Identification of Carbohydrate

Both preparations of taka-amylase A from "Takadiastase Sankyo" and taka-amylase from culture solution give positive Molisch's reaction, and the coloured materials obtained from Takadiastase or from the culture medium had the same behavior toward organic solvents (*9*) as that formed from carbohydrates. Carbohydrate contents of crystals of each crystallization step are shown in Table I.

TABLE I

Carbohydrate Contents in Various Recrystallization Steps

Recrystallization	Carbohydrate content (as glucose)
	%
1st. crystal	3.54
2nd. crystal	3.08
3rd. crystal	2.99

Reducing sugars in taka-amylase A were determined by micro-Bertrand's method. On heating the aqueous solution of taka-amylase A with Fehling's solution, greenish turbidity appeared, but no precipitate was detected by centrifugation and filtration. Consequently, it could be concluded that taka-amylase A contains no reducing sugar but it contains combined ones, probably in the form of *N*-glucosides or *O*-glucosides. The acid hydrolysate of taka-amylase A, which was obtained by heating 85 mg. of taka-amylase A with 0.5 *N* HCl at 100° for 1 hour, proved to contain 0.6 per cent reducing sugar by micro Bertrand's method. Orchinol-HCl and phloroglucinol tests for pentose were also positive.

For the purpose of confirming the above described results the authors studied the behaviour of carbohydrate on heating and acid denaturation of the enzyme protein.

Heat Denaturation—One ml. of 0.5 per cent taka-amylase A solution was added to 0.5 ml. of 1 *M* phosphate buffer of pH 6.0 and heated to 80° or 100° for 2 or 5 minutes and after cooling, it was centrifuged. The amounts of carbohydrate in the supernatant and the precipitate were determined by orcinol-H_2SO_4 method. The results are shown in Table II.

Acid Denaturation—To 1 ml. of 5 per cent taka-amylase A solution was added 1 ml. of 20 per cent trichloroacetic acid. After centrifugation the carbohydrate in the super-

natant and the precipitate was tested by Molisch's reaction. Only the latter showed an intensive colour reaction.

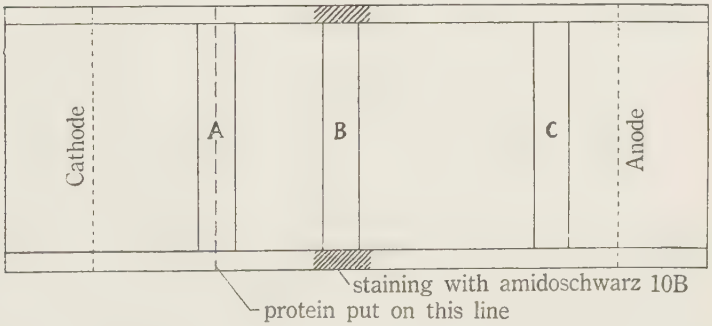
TABLE II

Carbohydrate Contents after Heat Denaturation of Taka-amylase A

Temperature	Time of heating	Optical density	
		Supernatant	Precipitate
°C	*min.*		
80	5	0.042	0.491
100	2	0.043	0.510
100	5	0.060	0.470

Paper Electrophoresis—A portion of 0.18 ml. of 15 per cent taka-amylase A in aqueous solution was subjected to electrophoresis on 15×40 cm. filter paper for 15 hours with 250 v-5 mA, using 0.1 *M* phosphate buffer of pH 7.0 as the buffer. After electrophoresis both edges of the filter paper were cut out and the zone of protein was detected by staining with Amidoschwarz 10 B (10). On the remaining filter paper, three separate parts A, B and C, each in 2 cm. wide, were cut as shown in Fig. 1 and eluted with water, and the quantities of carbohydrate in each fraction were determined by orcinol-H_2SO_4 method The results are given in Table III.

FIG. 1. Paper electrophoresis of taka-amylase A.



The further fractionation of taka-amylase A by alumina column chromatography was failed, and the intensities of carbohydrate reaction of each fractions run pararell to the protein contents as shown in Fig. 2.

Qualitative and Quantitative Analyses of Carbohydrate

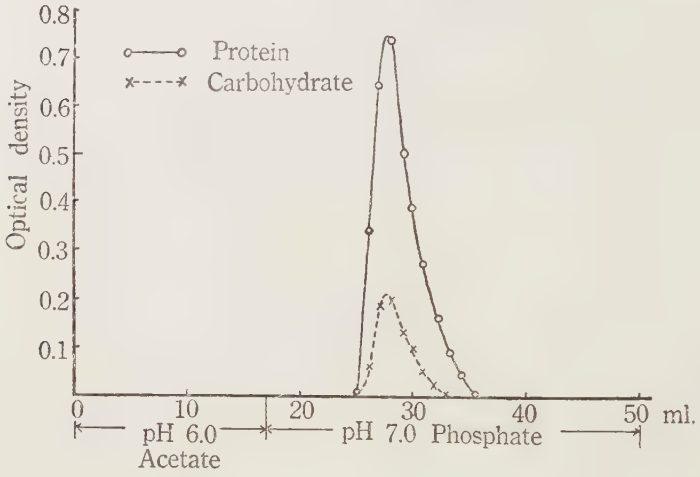
Separation of Carbohydrate from Amino Acid and Peptides—The nature of carbohydrate

TABLE III

Carbohydrate Distribution on the Paper after Paper Electrophoresis of Taka-amylase A

	Original point A	Protein Part B	Control C
Molisch's test	+	++	+
Pentose test	±	++	±
Optical density (by orcinol-H_2SO_4 method)	0.057	0.279	0.068

FIG. 2. Alumina column chromatography of taka-amylase A.



was determined by paper and starch column chromatographies. In order to obtain satisfactory result, removal of a large quantity of amino acids and peptides from acid hydrolysate was necessary. After several trials Amberlite IR-112 (H form) was found to be the most suitable for this purpose.

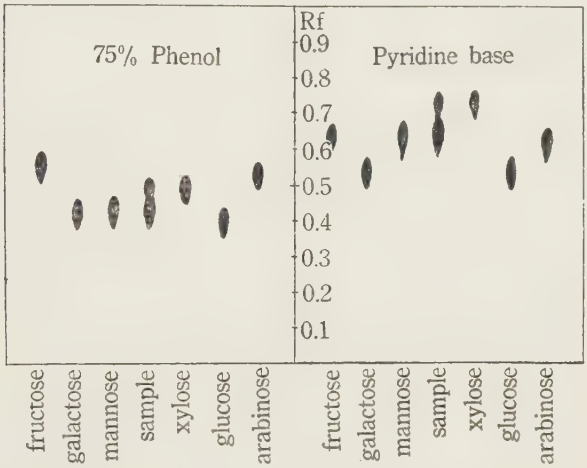
Amberlite IR-112 (Na form) of 60–200 meshes was washed with water, aqueous NaOH, and then with water and finally immersed in 4*N* HCl in a boiling water bath for 1 hour. After repeating this procedure, the resin was washed with water throughly, and poured into the column, which has 15 cm. high and 1.2 cm. diameter. By control experiments it was shown that all ninhydrin-positive materials were adsorbed from the solution to this resin and carbohydrates were passed through into the effluent without any substantial loss.

Taka-amylase A (500 mg.) was hydrolysed with 2*N* H_2SO_4 at 100° for 2 hours.

neutralyzed with 10 per cent baryta and centrifuged, and the precipitate was washed with hot water three times. The precipitate gave slightly positive Molisch's reaction. The supernatant was dried up, dissolved in 1~2 ml. of water and transferred to the column. The column was washed with 50 ml. of water, and the effluent, which contains carbohydrates, was dried up in vacuo.

Paper Chromatography—The fraction of carbohydrate, separated from amino acids and peptides, was subjected to one dimensional chromatography on filter paper. The solvent systems used were 75 per cent phenol and pyridine base. After development, the filter paper was dried at room temperature and stained with aniline-phthalate (*11*), whereby xylose could be detected clearly. On the other hand the separation of hexose was unsatisfactory. In Fig. 3 are shown the chromatograms of carbohydrates in taka-amylase A and that of standard carbohydrates.

Fig. 3. Paper chromatograpms of carbohydrates in taka-amylase A and of standard carbohydrates.



Column Chromatography—Recently, S. Gardell (*12*) succeeded to separate and determine monosaccharides on starch chromatography. Using this procedure the kind and ratio of amounts of monosaccharides in the hydrolysate of taka-amylase A were determined.

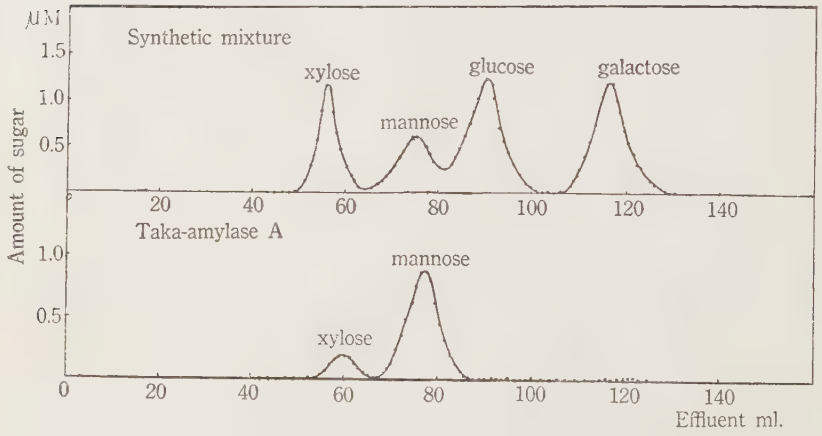
The carbohydrate fraction described above was placed on the starch column of 20 cm. high and 0.9 cm. in diameter, and developed with a solvent consisted with *n*-butanol, *n*-propanol and water (4:1:1, in volume). One ml. each of the effluent was collected with an automatic fraction collector, and the amount of carbohydrate in each fraction was determined by the method described by S. Gardell (*13*). The procedure was modified as follows.

The reagent solution was prepared by adding 2 ml. of aniline to the mixture of 50

ml. of *n*-butanol and 32 ml. of 8.5 *N*-trichloroacetic acid in an ice bath, stirring and diluting to 100 ml. with *n*-butanol. The effluent solution (1 ml.) was heated with 1 ml. of the reagent solution in a water bath at 80±1° for 30 minutes. After cooling by immersion in cold water 1 ml. of ethanol was added. The colour densities were measured by a photoelectric colorimeter using 1 cm. cell and 410 mμ filter. Pentose developes orange colour which gradually changes to yellow.

Diagrams in Fig. 4 represent the results obtained with a synthetic mixture of monosaccharides and with the carbohydrate fraction of taka-amylase A. From these experiments it is evident that carbohydrate in taka-amylase A consists of xylose and mannose and the ratio of xylose: mannose is approximately 1:10.

Fig. 4. Starch column chromatography of a synthetic mixture of monosaccharides and the carbohydrate fraction of taka-amylase A. Column: 0.9×20 cm., solvent: *n*-butanol, *n*-propanol and water (4:1:1).



The orcinol-H_2SO_4 method was used for the quantitative determination of carbohydrate of taka-amylase. For the standard solution of carbohydrate a mixture of xylose and mannose in 1 to 10 was used. The results were summarized in Table IV.

Hexosamine—Taka-amylase A showed positive hexosamine reaction as usual glucoproteins. Hexosamine content was determined by Elson and Morgan's method (*14*). The results are shown in Table IV.

To determine the kind of hexosamine, as well as its mode of combination in taka-amylase A molecule is the subject of further investigation.

TABLE IV

The Amount of Total Carbohydrate and Hexosamine in Taka-amylase A

Sample	Content of carbohydrate	Content of hexosamine
Taka-amylase A (I)	2.74%	0.74%
Taka-amylase A (II)	2.73	0.74
Taka-amylase from culture solution	0.25	—

(I) and (II) mean the crystals obtained from different batches of "Takadiastase Sankyo".

DISCUSSION

From the results of experiments described in this paper, it could be concluded that taka-amylase A obtained from "Takadiastase Sankyo" contains 8 moles of mannose, 1 mole of xylose and 2 moles of hexosamine per one molecule, assuming the molecular weight of the enzyme protein as 53,000 as reported previously (*1*). On the other hand, taka-amylase isolated from a synthetic culture solution contained the least amount of carbohydrate. The latter enzyme preparation was recrystallized only once, because the material was not sufficient for further purification. It could not be excluded, therefore, that carbohydrate found in taka-amylase from synthetic culture solution might be a contaminant.

The amylase activities per protein N of both preparations of taka-amylase were found to coinside with each other within experimental errors. Therefore, it may be concluded that the carbohydrate component is not essential to amylase activity.

As will be reported in succeeding paper (*15*), N-terminal amino acid of taka-amylase A obtained from "Takadiastase Sankyo" is alanine. Taka-amylase isolated from a culture solution has also only alanine as N-terminal amino acid. Nothing is known about the nature and site of combination between carbohydrate and protein, but the fact described above will suggest, at least, that the carbohydrate group is not combined with N-terminal amino group.

The authors wish to express their gratitudes to Sankyo Co., Ltd., for their kind supply of "Takadiastase Sankyo".

SUMMARY

Taka-amylase A obtained from "Takadiastase Sankyo" contains

3 moles of mannose, 1 mole of xylose and 2 moles of hexosamine as integral constituent of the molecule. Taka-amylase obtained from a synthetic culture solutions the least amount of carbohydrate. Carbohydrate component seems to be not essential to amylase activity.

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STUDIES ON XANTHURENIC ACID

XI. RESEARCH ON THE MECHANISM OF INITIAL HYPERGLYCEMIA CAUSED BY XANTHURENIC ACID

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(Received for publication, October 15, 1954)

As shown in the previous experiments (*1*, *2*, *3*) in this series of reports xanthurenic acid acts as diabetogenic agent to albino rat. In this case the blood sugar level passes usually 3 stages, namely an initial hyperglycemia, which occurs 3-4 hours after the injection, then a hypoglycemia and finally a second hyperglycemia, which appears 24 hours after the injection and lasts fairly long time.

Also an alloxan diabetes this initial hyperglycemia can be observed. The explantations for this fact by several investigaters are not quite the same.

Dunn *et al.*, (*4*) ascribed it either to insulin deficiency or to the increased decomposition of liver glycogen caused by stimulation of sympathetic nerve. Goldner and Gomiri (*5*) held the view that the initial hyperglycemia is in response to epinephrine, since the hyperglycemia could be inhibited either by ergotoxin injection or adrenalectomy. Kirschbaum, *et al.* (*6*), on the other hand, emphasized that in this case hypophysis is also responsible on the ground that such hyperglycemia did not occur in an animal with its hypophysis extirpated. Duff (*7*) and Okamoto (*8*) rather pointed out as its cause the interruption of insulin excretion from alloxan impaired β-cells of the Langerhans' islets. Goldner and Bailey (*9*) also suggested that, since it occurred even in a depancreatized animal, organs other than pancreas might concern with it. Houssay's (*10*) contention in this connection was that liver has more to do with it, being directly stimulated to lead to increased output of glucose. However, Kosaka (*11*) put forward the idea that not only the direct stimulation of liver but also some disturbance in the pituitary-adrenal system work together in giving rise to hyperglycemia.

As to the cause of hyperglycemia by xanthurenic acid no satisfactory explanation has been given. The author carried out various experiments to get clear idea on this point. Some interesting results were obtained, which will be reported here in details.

EXPERIMENTALS

Throughout the experiment albino rat of body weight 200 g. were used. In order to keep the nutritional condition]of the animals possibly constant they were fed for 2 weeks before the experiment on the diet consisting of casein 22 per cent, Mc Collum salts mixture 6 per cent, dried yeast 2 per cent, agar-agar 3 per cent, butter 10 per cent sucrose 5 per cent and starch 52 per cent, and housed in separate cage placed in a room of constant temperature 20°. Then the operations of extirpating suprarenal glands of both side or hypophysis were carried out on rat in the state of empty stomach. When the animals survived and regained normal appetite, they were submitted for the further treatments. Namely, to one group of rats were administered hypodermically each 1 ml. of 1000 × epinephrine solution per kg. of body weight and to another group 1 ml. of 20 per cent xanthurenic acid intraabdominally.

Then the change of blood sugar was followed with times as indicated in the table on samples collected from the tail vein, using the King and Garner's (*12*) true sugar determination method.

At the same time, the behaviours of liver glycogen in above operated rats were studied and compared with those of the injected (unoperated) and also with that of the normal. The determination of glycogen was done on a slice cut out from marginal part of left liver lobe of animal which was brought to death by bleeding from carotid artery. Further, on the animals, whose suprarenal glands remained untouched, ascorbic acid contents in the glands were determined with the purpose to get some in sight in the attitude of ACTH under such treatments.

The experimental results are summarized in Tables I and II where the average figures of a number of experiment are given.

It is noted that liver glycogens of rats operated or injected with xanthurenic acid were reduced nearly to half of the normal value, and that of epinephrine treated rat to less than one third.

From Table II it is worth to notice that hyperglycemia due to epinephrine was not so apparent. More remarkable is the fact that xanthurenic acid did not cause any rise but rather abrupt fall of blood sugar in rats deprived of suprarenal glands. In the case of hypophysectomised animal, xanthurenic acid also caused a lowering of blood sugar, but in less degree than in the former case. Although the reduced glycogen storage in such operated animals has some concerns in surpressing the rise of blood sugar, these results clearly indicate that for the appearance of initial hyperglycemia by xanthurenic acid the presence of suprarenal glands and of hypophysis is necessary.

One may well imagine, therefore, that such hyperglycemia induced by xanthurenic acid might be caused by stimulating sympathetic nerve prompting the tempolary over-

TABLE I

The Liver Glycogen and Suprarenal Gland Ascorbic Acid of Variously Treated and Normal Rats

Treatment	Cases	Liver glycogen amount	Ascorbic acid amount in suprarenal gland
		g. %	*mg. %*
Suprarenal-glands-extirpated group	8	0.909	
Hypyphysis-extirpated group	5	0.987	
Epinephrine-injected group	5	0.508	209.7
Xanthurenic-acid-injected group	5	0.933	246.3
Normal group	10	1.823	261.7

TABLE II

Blood Sugar of Variously Treated Rats

Treatment	Cases	*hrs.* 0	1/1	1	1½	2	3	4
I. Epinephrine-injected group with suprarenal glands extirpated	4	56.8	62.1	87.6	74.6	51.4	48.6	
		mg./dl.						
II. Xanthurenic acid-injected group with suprarenal glands extirpated	10	65.7	62.5	39.3	24.0	24.1	32.0	35.5
III. Xanthnrenic acid-injected group with hypophysis extirpated	5	61.3	61.6	61.6	55.0	50.6	41.5	47.3

production of epinephrine.

To raise any additional evidence in this regard, the ffect of benzyl-imidazolin was studied. This preparation has the activity to paralyse sympathetic nerve functions.

One ml. of its 2 per cent solution per kg. of body weight was administered together with epinephrine or xanthurenic acid of same dosis as used in the foregoing experiments on normal rats and then blood sugar, liver glycogen and ascorbic acid were determined. The results are summarized in Table III.

It can be seen from Table III that epinephrine and xanthurenic acid alone produced hyperglycemia accompanied with the reduction of liver glycogen and ascorbic acid in suparenal glands. But when benzyl-imidazolin was applied together with epine-

TABLE III

The Effect of Simultaneous Application of Benzyl-imidazolin with Epinephrine and xanthurenic Acid on Blood Sugar, Liver Glycogen and Suprarenal Gland Ascorbic Acid

Treatment	Blood sugar amount *mg./dl.*						Liver glycogen amount	Ascorbic acid amount in suprarenal gland
	hrs. 0	0.5	1.0	1.5	2.0	3.0	*g.%*	*mg.%*
Epinephrine injected	56.6	112.8	154.2	119.8	108.7		0.508	209.7
Xanthurenic acid injected	69.7	103.0	108.5	103.7	104.4	141.9	0.933	246.3
Epinephrine+benzyl-imidazolin	61.8	76.1	62.8	40.2	28.1		1.527	331.9
Xanthurenic acid+ benzyl-imidazolin	75.0	97.8	97.1	112.7	105.2	96.6	1.012	330.4
Benzyl-imidazolin	70.3	78.1	73.2	65.7	54.8		1.627	361.9

phrine no more hyperglycemia and such reduction of liver glycogen was observed.

In the case of xanthurenic acid, however, the matter was somewhat different. Here benzyl-imidazolin did not supress the rise of blood sugar and the decrease of liver glycogen due to xanthurenic acid, although the decrease of ascorbic acid was not ascertained. This result was quite unexpected.

This finding leads the author's to believe that xanthurenic acid acts not only indirectly through epinephrine but also directly on liver.

DISCUSSION

The intimate concern of pituitary hormons to diabetes was first introduced by Houssay (*13*) and substanciated by Young (*14*), who succeeded in producing experimentally glycosuria by repeated injection of the hormons.

Later the so-called "Stress" theory was forwarded by Selye (*15*) in which he assumed that a corticotropic hormon ACTH produced in pituitary gland gives rise to corticoid in suprarenal gland, and that corticoid exhibits diabetogenic action by the increased production of glucose, decreased glucose torelance and increased resistance against insulin. In this regard the role of epinephrine is understood to stimulate the output of corticotropic hormon from hypophysis. As to xanthurenic acid, its action stands at the same category with epinephrine, since in

absence of suprarenal gland or hypophysis no more hyperglycemia was observed. Further the decrease of ascorbic acid in suprarenal glands of normal rats rendered hyperglycemia by injection of xanthurenic acid might be taken as an additional evidence, because xanthurenic acid is assumed to work as a sort of " Stressor ", the characteristic of which is to induce increased output of corticoid through ACTH accompanied by the decrease of ascorbic acid in suprarenal gland.

Aside from the above considerations it should be mentioned that the initial hyperglycemia by xanthurenic acid may also be caused by the direct action of the acid on liver tissue. This position is based on the finding that simultaneous application of benzyl-imidazolin with xanthurenic acid did not stop hyperglycemia as in the case of epinephrine. Since benzyl-imidazolin is an agent to block the function of sympathetic nerve system, the result should be interpreted as indicating that there is another role than hormonal, namely xanthurenic acid—epinephrine—ACTH—corticoid.

As there are a lot of evidences that xanthurenic acid plays important roles in catabolic and anabolic processes in liver, so it may be concluded that the acid gives a rise to hyperglycemia acting directly on liver tissue.

SUMMARY

1. On albino rats with their hypophysis or suprarenal gland extirpated the initial hyperglycemia by xanthurenic acid did not appear. In these cases the liver glycogen contents were found invariably reduced by half as compared with normal ones.

2. Following the injection of xanthurenic acid, the ascorbic acid amount in the suprarenal gland was found reduced just as in the case of epinephrine injection.

3. Hyperglycemia due to epinephrine was inhibited by benzyl-imidazolin, but not that due to xanthurenic acid.

4. These experimental facts lead us to believe that the initial hyperglycemia caused by the injection of xanthurenic acid is due partly to hormons of suprarenal glands and hypophysis, the production of which being enhanced by the injected acid, and partly to its direct action on liver tissue.

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ON THE MECHANISM OF THE ACTIVATION OF α-CHYMOTRYPSINOGEN TO α-CHYMOTRYPSIN

II. THE CHANGES IN AMINO-TERMINAL AMINO ACIDS OF α-CHYMOTRYPSINOGEN DURING THE ACTIVATION BY TRYPSIN

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(Received for publication, October 19, 1954)

The activation of α-chymotrypsinogen by trypsin has been regarded as a complicated process, proceeding through two intermediates, called π-chymotrypsin and δ-chymotrypsin, successively, and furnishing a mixture of several chymotrypsins designated by the letters α, β, and γ (*1*, *2*). In the previous work (*3*), α-chymotrypsinogen was activated by the method of Northrop and Kunitz (*1*) so as to produce mainly α-chymotrypsin, and studied electrophoretically, whereby the appearance of 12 components of relatively high molecular weights was confirmed in the course of the activation in 24 hours. It was thus concluded by the author that the activation process of α-chymotrypsinogen to α-chymotrypsin is not a simple process as previously supposed, but a highly complex one.

In the present work α-chymotrypsinogen was activated by the method described in the previous report (*3*), and the changes in amino-terminal (N-terminal) amino acids in α-chymotrypsinogen during the activation process were investigated to elucidate the correlation between proteolytic activity and N-terminal group. N-terminal amino acids of α-chymotrypsin were also determined to compare with those of diisopropylphosphoryl (DIP)- α-chymotrypsin.

EXPERIMENTAL

α-Chymotrypsinogen—α-Chymotrypsinogen was prepared from fresh beef pancreas by the method of Kunitz *et al.* (*4*). It was crystallized thrice with ammonium sulfate. The final crystals were found to be electrophoretically monodisperse, and gave only one ninhydrinpositive spot on the paperchromatogram.

Trypsin—Trypsin was prepared from the mother liquor of crystalline α-chymotryp-

sinogen by self activation followed by crystallization by the method of Kunitz *et al.* (*4*). Its specific activity was 0.24 $[T.U.]^{cas.}_{mg.p.N.}$.

Activation of α-Chymotrypsinogen—Crystalline α-chymotrypsinogen filter cake (2 g.) was suspended in 5.2 ml. of water and dissolved by adding several drops of 5 *N* sulfuric acid, and then 2 ml. of *M*/2 phosphate buffer (pH 7.6), and a quantity of molar sodium hydroxide equivalent to the acid were added. The mixture was cooled to 5°, and activated for 4 days at the same temperature by addition of 0.8 ml. of trypsin solution containing 0.9 mg. of trypsin protein. Aliquots were removed at various time intervals and their proteolytic activities were determined by the method described in the previous report (*3*). The results are shown in Fig. 1.

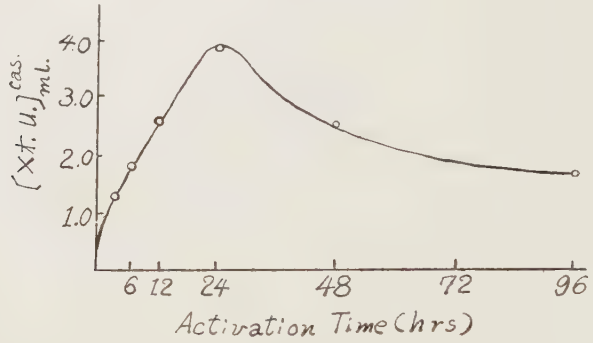

Fig. 1. The proteolytic activity during the activation.

At the same time, the amino acids possessing free α-amino group in the solution were characterized and estimated quantitatively by dinitrophenyl (DNP) method of Sanger (*5*).

The Changes in N-terminal Amino Acids of α-Chymotrypsinogen during Activation Process—A 0.5 ml. aliquot was mixed with 1 ml. of half saturated sodium bicarbonate solution and 3 ml. of 10 per cent alcoholic solution of dinitrofluorobenzene (DNFB) and shaken mechanically for 3 hours. Then the solution was desiccated in vacuum to dryness and dissolved with 3 ml. of water and shaken thrice with 6 ml. of ether for removal of excess DNFB. The aqueous solution was neutralized with 2 *N* hydrochloric acid, and then mixed with an equal volume of concentrated hydrochloric acid and hydrolyzed for 10 hours at 100° in a sealed tube. DNP-amino acids in the hydrolyzate were extracted 5 times with 10 ml. of ether, and characterized and estimated according to the method of Sanger (*5*).

As α-chymotrypsin has two N-terminal groups, *i.e.* alanine and isoleucine*, and both tryptic and chymotryptic hydrolysis should not give water soluble DNP-amino

* It will be discussed later.

acids, so that DNP-amino acids determined in these conditions should represent all the N-terminal amino acids of α-chymotrypsin, as well as intermediate proteins or liberated polypeptides. The results are shown in Table I and Fig. 2.

TABLE I

N-Terminal Amino Acids of α-Chymotrypsinogen during the Activation Process

	N-terminal amino acids* in moles per mole α-chymotrypsinogen						
Activation time *hours*	0	3	6	12	24	48	96
Aspartic acid	0.05	0.25	0.31	0.28	0.27	0.30	0.30
Serine	0.04	0.47	0.48	0.49	0.49	0.50	0.50
Threonine		0.47	0.50	0.56	0.72	0.75	0.82
Glycine		0.36	0.37	0.40	0.43	0.47	0.45
Alanine	0.04	0.65	0.65	0.70	0.71	0.78	0.80
Phenylalanine			0.17	0.23	0.21	0.25	0.39
Leucine (or Isoleucine)		0.32	0.35	0.48	0.57	0.60	0.74
Total	0.13	2.52	2.83	3.14	3.40	3.65	4.00

* Not corrected for the decomposition of DNP-amino acids during acid hydrolysis, and the molecular weight of α-chymotrypsinogen is assumed to be 25,000 (6).

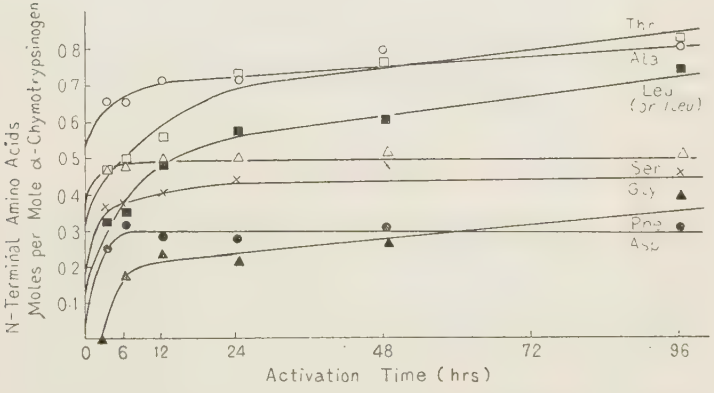

FIG. 2. N-terminal amino acids of α-chymotrypsinogen during the activation process.

N-Terminal Amino Acids of α-Chymotrypsinogen, α-Chymotrypsin and DIP-α-Chymotrypsin—Crystalline α-chymotrypsin was prepared from α-chymotrypsinogen according to the method of Kunitz *et al.* (*4*), and crystalline DIP-α-chymotrypsin was prepared from α-chymotrypsin by the method of Jansen *et al.* (*7*).

N-terminal amino acids of these three crystalline proteins were characterized and estimated according to the method of Sanger (*5*), and the results are shown in Table II.

TABLE II

N-Terminal Amino Acids of α-Chymotrypsinogen, α-Chymotrypsin and DIP-α-Chymotrypsin

	N-terminal amino acids* in moles per mole protein		
Crystalline proteins	α-Chymotrypsin	DIP-α-chymotrypsin	α-Chymotrypsinogen
Aspartic acid	0.10	0.10	0.08
Serine	0.23	0.07	
Threonine	0.08	0.10	0.21
Glycine	0.44		0.07
Alanine	1.10	1.13	0.10
Phenylalanine	0.44		
Leucine (or Isoleucine)	0.78	0.85	

* Corrected for the decomposition of DNP-amino acids during acid hydrolysis, and the molecular weights of each of these three proteins are assumed to be equally 25,000 (*7*).

DISCUSSION

Since the activation of α-chymotrypsinogen to α-chymotrypsin is mediated by trypsin, and is accompanied by spontaneous or autocatalytic hydrolysis of intermadiate proteins as well as the increase of nonprotein nitrogen (*1, 2, 3, 8*), α-chymotrypsin should differ from α-chymotrypsinogen in the number or nature of terminal groups of the constituent polypeptide chains or in both. Accordingly, the present experiments have been carried out in order to compare the N-terminal groups of the zymogen before and after activation.

It is shown that α-chymotrypsinogen is presumably devoid of N-terminal groups, because a detectable amount of N-terminal amino acids is absent as shown in Table II. On the other hand, DIP-α-chymotrypsin has about 1 equivalent each of alanine and leucine (or isoleucine) with negligibly small amount of several other amino acids as N-terminal groups, whereas α-chymotrypsin has 0.4 equivalents each of glycine and phenylanine in addition to one mole equivalent each of alamine and leucine (or isoleucine). Accordingly, if the two following assumptions are accepted (see below), it may be concluded that α-chymotrypsin has two N-terminal groups, *i.e.* alanine and leucine (or isoleucine), as already proposed by Desnuelle and his coworkers (*9*).

1. α-Chymotrypsin and its DIP derivative are structurally identical (*7*), except that the latter contains a diisopropyl phosphate group attached to a non-terminal group.

2. The other N-terminal groups than alanine and leucine (or isoleucine) can be attributed to the contaminated proteins or peptides which had been absorbed to or combined with α-chymotrypsin.

Recently, Gladner and Neurath (*10*) have investigated the effect of carboxypeptidase on α-chymotrypsinogen and DIP-α-chymotrypsin in an effort to determine by enzymatic means the carboxyl-terminal groups of these proteins, and concluded that the activation process of α-chymotrypsinogen by trypsin involves the opening of cyclic polypeptide chains of the zymogen, giving rise to leucine and tyrosine as carboxyl-terminal groups of α-chmotrypsin and a basic peptide.

Therefore, it may be admitted that by means of activation, α-chymotrypsinogen composed of two cyclic polypeptides without terminal groups, may be changed into α-chymotrypsin with two amino-terminal groups and two carboxyl-terminal groups. Although the cleavage of one peptide bond in each of the two cyclic polypeptide chains seems to explain the above facts, but it will not be able to explain the previous experimental results (*3*) as well as the present ones which would lead to the following interpretations.

1) Seven kinds of N-terminal amino acids, aspartic acid, serine, threonine, glycine, alanine, phenylalanine, and leucine (or isoleucine) were detected in the activation process. As alanine and leucine (or isoleucine) were found to be the two N-terminal groups of α-chymotrypsin, the other five amino acids may be N-terminal groups of intermediate proteins or liberated peptides.

2) The proteolytic activity in the activation mixture increases almost lineally for 24 hours and subsequently decreases to half of the

maximum activity at the 24th hour. But the total amount of N-terminal amino acids detected increases reapidly in th first 3 hours and then gradually during the whole activation course.

The increase in the total amount of N-terminal amino acids from the 24th hour to the 96th hour seems not to be enough to explain the decrease of the proteolytic activity during that time. Moreover, each of N-mterinal amino acids detected during the activation does not amount to 0.5 moles per mole of α-chymotrypsinogen, except alanine, leucine (or isoleucine), and threonine. Therefore, the activation of α-chymotrypsinogen to α-chymotrypsin may also involve the liberation of peptides, transpeptidation, secondary structural modification and so on.

3) In Fig. 2, alanine, one of the two N-terminal groups of α-chymotrypsin, appears at the third hour for the most part. In contrast, leucine (or isoleucine), another N-terminal group, appears slowly in the course of the activation.

Therefore, one of the two cyclic polypeptide chains in α-chymotrypsinogen seems to be opened at the initial step of the activation process.

SUMMARY

1. The changes in N-terminal groups of α-chymotrypsinogen during the activation has been investigated, and seven kinds of N-terminal amino acids were detected for the most part at the initial stage of the activation process.

2. The activation process of α-chymotrypsinogen to α-chymotrypsin by trypsin involves the opening of cyclic polypeptide chains of the zymogen, yielding alanine at first and then isoleucine as amino-terminal groups of α-chymotrypsin, and it may be accompanied with the liberation of peptides, transpeptidation, secondary structural modification, and so on.

The author wishes to thank Professor Shiro Akabori for his guidance throughout of this work, also Mr. Yoshimi Okada for his valuable technical assistance.

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The Journal of Biochemistry, Vol. 42, No. 1, 1955

THE SYNTHESIS OF STERO-BILE ACIDS.

XII. THE PARTIAL SYNTHESIS OF BISNORSTEROCHOLIC ACID BY KOLBE ELECTROLYTIC REACTION

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(Received for publication, October 26, 1954)

The various stero-bile acids having more number of carbon atoms than the normal bile acid has been isolated from animal biles (*1*, *2*, *3*). For the purpose to clear up their chemical constitution, the authors have already synthesized some of these natural stero-bile acid derivatives and further those which have hitherto been unknown in nature. In this laboratory the following stero-bile acids and neutral steroids have so far been synthesized, *i.e.*

1) The neutral steroids with longer hydrocarbon side chains;

trihydroxynorcholane $C_{23}H_{40}O_3$ ($R=-\overset{\overset{CH_3}{|}}{CH}-CH_2-CH_3$) (*4*), trihydroxycholane $C_{24}H_{42}O_3$ ($R=-\overset{\overset{CH_3}{|}}{CH}-CH_2-CH_2-CH_3$) (*5*), tryhydroxyhomocholane $C_{25}H_{44}O_3$ ($R=-\overset{\overset{CH_3}{|}}{CH}-CH_2-CH_2-CH_2-CH_3$) (*5*), trihydroxybisnorsterocholane $C_{26}H_{46}O_3$ ($R=-\overset{\overset{CH_3}{|}}{CH}-CH_2-CH_2-CH_2-CH_2-CH_2-CH_3$) (*6*), trihydroxynorsterocholane $C_{27}H_{48}O_3$ ($R=-\overset{\overset{CH_3}{|}}{CH}-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_3$) (*7*), and trihydroxycoprostane $C_{27}H_{48}O_3$ ($R=-\overset{\overset{CH_3}{|}}{CH}-CH_2-CH_2-CH_2-\overset{\overset{CH_3}{|}}{CH}-CH_3$) (*8*),

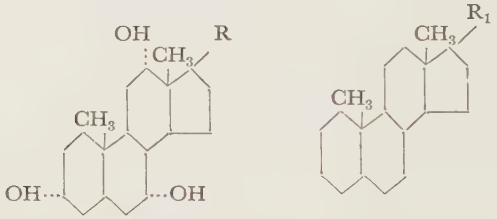
2) The stero-bile acids;

bisnorcholic acid $C_{22}H_{36}O_5$ ($R=-\overset{\overset{CH_3}{|}}{CH}-COOH$) (*9*), norcholic acid $C_{23}H_{38}O_5$ ($R=\overset{\overset{CH_3}{|}}{CH}-CH_3-COOH$) (*9*), homocholic acid C_{25}-

$H_{42}O_5$ ($R=-\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_2-CH_2-COOH$) (*6*), norsterocholic acid $C_{27}H_{46}O_5$ ($R=-\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_2-CH_2-CH_2-CH_2-COOH$) (*10*), and trihydroxycoprostanic acid $C_{27}H_{46}O_5$ ($R=-\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_2-CH_2-\underset{\underset{CH_3}{|}}{CH}-COOH$) (*11*),

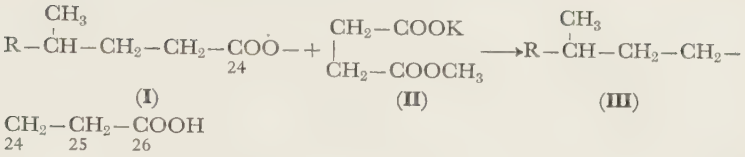
3) The hydroxy-free derivatives of the stero-bile acids and steroids ;

cholane $C_{24}H_{42}$ ($R_1=-\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_2-CH_3$) (*5*), 24-methyl-bisnorsterocholanic acid $C_{27}H_{46}O_2$ ($R_1=-\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_2-\underset{\underset{CH_3}{|}}{CH}-CH_2-COOH$) (*12*), and sterocholanic acid $C_{28}H_{48}O_2$ ($R_1=-\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_2-\underset{\underset{CH_3}{|}}{CH}-\underset{\underset{CH_3}{|}}{CH}-COOH$) (*13*).



It has been determined that among these synthesized stero-bile acids or steroids, trihydroxyhomocholane and trihydroxycoprostanic acid, respectively, were identical with trihydroxy-steroid melting at 186° (*14*) and sterobile acid melting at 195° isolated from Rana nigromaculata bile (*15*), and the synthetic sterocholanic acid was identical with the stem acid of trihydroxy-bufosterocholenic acid (*16*), one of the toad bile component having 28 carbon atoms and cholic acid nucleus. Except for these substances synthesized stero-bile acids or steroids have hitherto never been discovered in nature.

In this report, one of the stero-bile acids, bisnorsterocholic acid $C_{26}H_{44}O_5$ (**III**) was synthesized from potassium cholate (**I**) and methyl hydrogen succinate (**II**) by the application of the Kolbe electrolytic reaction.



EXPERIMENTAL

Methyl Bisnorsterocholate:

Eleven g. of methyl hydrogen succinate, 1.4 g. of potassium carbonate, 3 g. of potassium cholate and 6 g. of cholic acid were dissolved in 150 ml. of methanol and this solution was electrolyzed for 7 hours with electric current of 25 volt and 0.6–0.7 ampere by the same method described in the prev ous report (*5*), but distance of the electrode was shortened to 0.3 cm. At a half way of electrolysis, 7 g. of methyl hydrogen succinate and 2 g. of cholic acid were added to the electrolyzing solution and neutralized. When the electrolysis was ended, the solution was neutralized with acetic acid, methanol was evqporated under reduced pressure, and the residues were extracted with ether. The ether solution was washed with sodium carbonate solution and then with water, dried, and the ether was distlled off. The residue was hodrolyzed with 8 per cent methanolic potassium hydroxide for three hours, diluted with water and ethanol was evaporated. Acidification of the alkaline solution with dilute hydrochloric acid resulted in precipitation. It was separated, dried and esterified with ethereal diazomethane. By evaporation of ether 2.2 g. of solid residue were obtained. It was dissolved in benzene and chromatographed over 70 g. of alumina. From the eluate of ether-acetone and acetone, a crystal was obtained. The crystal was recrystallized from benzene-petroleum ether and then from methanol-water. 600 mg. of needle like crystal with m.p. 150–152° were obtained.

Analysis: Calcd. for $C_{27}H_{46}O_5$: C 72.00; H 10.22
Found: C 71.92; H 10.16.

Bisnorsterocholic acid:

Five hundred mg. of ethyl bisnorsterocholate was hydrolyzed with 8 per cent methanolic potassium hydroxide for three hours and diluted with water, and then methanol was evaporated. The alkaline solution was acidified with dilute hydrochloric acid. The precipitate was recrystallized from methanol water and 350 mg. of needle like crystal with m.p. 192–3° were obtained. Hammarsten's and Liebermann's reaction were both positive.

Analysis: Calcd. for $C_{26}H_{44}O_5$: C 71.56; H 10.22
Found: C 71.23; H 10.45.

Dehydrobisnorsterocholic acid was prepared from the above acid by chromic acid oxidation in glacial acetic acid. Crystallization from methanol gave a prism with m.p. 223–225°.

This work was supported by a Grant in Aid for Fundamental Scientific Research from Department of Education.

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STUDIES ON THE ANTERIOR PITUITARY ACTH

III. CHROMATOGRAPHY OF BEEF ACTH ON ION EXCHANGE RESIN COLUMN

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(Received for publication, November 18, 1954)

Some years ago, Li and his collaborators (*1*) showed that ACTH activity survived, in part at least, after the treatment of protein hormone with pepsin or acid under definite conditions; this led to an intensive search for peptides with ACTH activity. In an attempt to franctionate a mixture of peptides produced by acid or pepsin hydrolysis, it was shown that ACTH active material might be basic in character by some experiments, *e.g.* electrodialysis. According to Astwood *et al.* (*2*), ACTH activity could be adsorbed on powdered cellulose or oxycellulose from dilute acetic acid solution and eluted again with 0.1 *N* HCl. Such behavior agreed also with the postulate that the active factor might be basic in nature.

The basic proteins, lysozyme and ribonuclease had been isolated by means of resolution of a column of an ion exchange resin. Dixon *et al.* (*3*) tried to separate the active peptides of ACTH on a column of an ion exchange resin and was able to separate from the pig ACTH protein a fraction which contained most of the biological activity in a portion yielding only 5 per cent of the total ninhydrin colour.

The present paper describes the separation of beef ACTH by an ion exchange resin. The beef ACTH, prepared according to the method of Astwood *et al.* (*4*) was adsorbed on the column of the carboxylic acid ion exchange resin Amberlite IRC-50 at pH 7.8 and eluted the active substance with organic acid. The effluent was collected by fraction collector and its protein concentration was estimated with Folin reagent (*5*).

When eluted with formic acid, the concentration of which is increased from 0 per cent to 25 per cent gradually, four peptide fractions were obtained, but activity was scarcely found only in the 1st fraction. It seemed that formic acid will denaturate the ACTH and destroy its hormonal activity.

When acetic acid (0–35 per cent) was used as eluent, in this case 4 main frctions were obtained and the 2nd fraction which eluted in acid concentration from 12 per cent to 18 per cent contained most of the active substance. The activity of this fraction is about 20 fold as great as original ACTH.

EXPERIMENTAL

Material and Method—Beef ACTH was prepared by the method of Astwood *et al.* (*4*) which involves extraction of the acetone-dried beef pituitary gland with glacial acetic acid-acetone mixture at 70° followed by acetone and ether precipitation.

Chromatography was carried out on the column of Amberlite IRC-50 and to convert a column of resin to a mixed salt-acid form, a phosphate buffer solution was employed. The treatment involved first converting the resin to the Na form with 4 per cent NaOH, washed with water followed by treatment with the buffer solution at a low rate of flow.

Definite volume of the effluent was collected by fraction collector and used for estimation of protein quantity, acid concentration and hormonal activity. Quantitative protein analysis of effluent fraction was carried out with the modified Folin reagent (*5*). ACTH activity was determined by the method of Sayers *et al.* (*6*).

Elution with Formic Acid—Six g. of Amberlite IRC-50 resin was prepared according to the procedure of Stein *et al.* (*7*) (1 × 16 cm. column, final volume 12 ml.). For the adsorption of ACTH, the resin was buffered at pH 7.8 with phosphate buffer.

ACTH solution (200 mg. in 20 ml. distilled water) was passed through the column at the rate of 0.1 ml. per minute. The unadsorbed solution was assayed by Sayers' method, but no activity was found. The column was washed with water, then eluted with formic acid (0.1 ml. per minute). The concentration of acid was increased from 0 per cent to 25 per cent gradually. The effluent was collected by fraction collector (1 fraction = 1.2 ml.) and protein concentration was determined by the modified Folin reagent (*5*). The result was shown in Fig. 1.

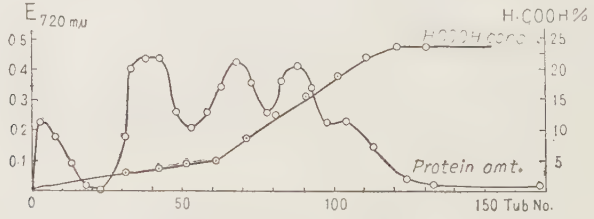

Fig. 1. Chromatography of beef ACTH on Amberlite IRC-50 eluted with HCOOH (0–25%)

In this experiment, 4 main fractions were obtained as follows:

Fraction A (Tube No. 1–20), eluted with 0–2% HCOOH
„ B (Tube No. 25–52), „ „ 2–4% „
„ C (Tube No. 53–76), „ „ 4–11% „
„ D (Tube No. 77–98), „ „ 11–17% „

To estimate the hormonal activity of each fraction, the main part of the fraction was precipitated with acetone and 25 μg. of precipitate was used for assay. As shown in Table I, the hormonal activity was slightly found in fraction A, but other fractions have no activity. In another experiment, thioglycol was added to the formic acid, but activity was not found in any fraction.

Elution with Acetic Acid—A 1.5×21.3 cml column of Amberlite IRC-50 (final volume of resin, 47 cc.) was prepared as above and 50 ml. of 2 per cent ACTH solution was passed through the column. Elution of the column was performed with acetic acid, the concentration of which was increased from 0 per cent to 35 per cent gradually. Other experimental conditions were the same as above. The result was shown in Fig. 2.

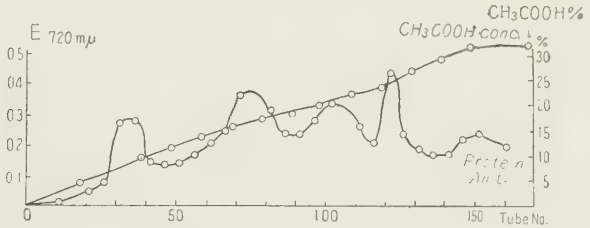

Fig. 2. Chromatography of beef ACTH on Amberlite IRC-50 eluted tion with CH_3COOH (0–35%)

In this case 4 main fractions and 2 smaller fractions were obtained as follows:

Fraction A (Tube No. 11–41), eluted with 0–12% CH_3COOH
„ B (Tube No. 50–85), „ „ 12–18% „
„ C (Tube No. 90–115), „ „ 18–23% „
„ D (Tube No. 115–130), „ „ 23–27% „

The hormonal activity of each fraction was assayed as above, and the result was shown in Table I. Most of the hormonal substance were concentrated in fraction B and activity of this fraction was *ca.* 20 fold as active as compared with original ACTH. Fraction A and C also contained little activity, but it seemed to come from the contamination of fraction B. From these experiments, it was demonstrated that the beef ACTH was also adsorbed on Amberlite IRC-50 and was eluted specifically with acetic acid

TABLE I

Hormonal Activity of Fractions Obtained from Ion Exchange Resin Chromatography

Hormone fraction	Dose	Ascorbic acid* depletion rate	Activity**
Original beef ACTH	500 μg.	−19.8%	1.0
HCOOH elution, Frac. A	25	−3.1	—
,, ,, B	,,	−1.2	—
,, ,, C	,,	+2.5	—
,, ,, D	,,	+0	—
CH_3COOH elution, Frac. A	,,	−10.7	2.0
,, ,, B	,,	−22.3	20.0
,, ,, C	,,	−6.1	1.0
,, ,, D	,,	+2.7	—

* Ascorbic acid depletion rate was shown as next equation.

$$R = \frac{\text{ascorbic acid of left adrenal} - \text{ascorbic acid of right adrenal}}{\text{ascorbic acid of left adrenal}}$$

Ascorbic acid content was expressed by mg. per 100 g. adrenal.

** Original ACTH was posturlated as 1.0.

SUMMARY

Beef ACTH was adsorbed on Amberlite IRC-50 at pH 7.8 and could be eluted with acetic acid, but foric acid destroyed its hormonal activity. Elution with acetic acid increased hormonal activity about 20 folds.

We wish to express our gratitude to Prof. S. Akabori, Osaka University and Dr. K. Takeda for their kind guidance and encouragements through this work, and to Mr. A. Tanaka for his much technical assistance.

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The Journal of Biochemistry, Vol. 42, No. 1, 1955

STUDIES ON THE ANTERIOR PITUITARY ACTH

IV. ACID HYDROLYSIS OF BEEF ACTH

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(Received for publication, November 18, 1954)

It is well known that ACTH is comparatively stable in acid solution. C.H. Li (*1*) studied the stability of sheep ACTH in acid solution and found that by heating at 100° in 1 *N* HCl solution, the protein was hydrolyzed to 52 per cent in 25 minutes, but retained its hormonal activity perfectly. When heated more time, the hormonal activity decreased gradually and at 80 minutes the protein was hydrolyzed to 91 per cent, although its activity still retained to some extent. He also showed that these active fractions were soluble in 5 per cent trichloroacetic acid solution. Heating for 120 minutes, the protein was hydrolyzed perfectly and its activity also vanished. At this point, the free amino-N increased from 0.8 per cent to 5.1 per cent indicating that a definite hydrolysis of the peptide chains occured, but only a small amount of free amino acid was liberated.

Sayers *et al.* (*2*) studied with pig ACTH and found that it lost 40 per cent of the activity in 1 *N* HCl solution at 100° for 30 minutes.

The authors studied this partial hydrolysis of beef ACTH and found that heating for 60 minutes in 1 *N* HCl solution at 100°, beef ACTH protein was split to 70 per cent, but retained its hormonal activity about a half. When heating at 100° for 90 minutes, its activity was destroyed perfectly. The active fraction of partial hydrolyzate of beef ACTH was precipitated with 5 per cent trichloroacetic acid solution. The paper electrophoresis and N-distribution were also studied on these samples.

EXPERIMENTAL

Material and Method—Original ACTH was prepared from acetone dessicated powder of beef anterior pituitary by the method of Astwood *et al.* (*3*).

A 2 per cent solution of beef ACTH in 1 *N* HCl was heated at 100° at various time intervals and its degree of hydrolysis and its biological activity was studied.

The hormonal activity was estimated by ascorbic acid depletion method of Sayers *et al.* (*4*).

Partial Hydrolysis for 30 Minutes—One g. of beef ACTH was added to 50 ml. of 1 *N* HCl and heated at 100° for 30 minutes. After cooling the insoluble residue was centrifuged off and soluble fraction was concentrated to 10 ml. *in vacuo* and poured into 200 ml. of cold acetone. After storing in refrigerator overnight, the precipitate was centrifuged and washed with acetone and dried *in vacuo*. Yield, 260 mg. (ACTP–1).

The hormonal activity of this sample was slightly higher than that of original material.

Partial Hydrolysis for 60 Minutes—Partial hydrolysis was done as above for 60 minutes. From 100 mg. ACTH 44 mg. of yellowish brown powder was obtained as HCl soluble fraction (ACTP-2). The activity of this sample was less than that of original material.

Hydrolysis for 90 Minutes—In the case of 90 minutes heating, 55 mg. of HCl soluble fraction was obtained from 100 mg. ACTH (ACTP-3), but this sample showed no corticotropic activity with 500 μg.

Separation of Active Fraction of ACTP–1 with 5 per cent Trichloroacetic Acid—A portion of 100 mg. of ACTP–1 was dissolved in 5 ml. of 0.01 *N* acetic acid and added 5 ml. of 10 per cent trichloroacetic acid (TCA) solution. The precipitate was centrifuged and the supernatant was shaken with acidified ether several times, then TCA was perfectly freed from the solution with ion exchange resin Amberlite IRA-400.

The solution was concentrated in vacuo and precipitated with acetone. Thus obtained 45 mg. of white powder (ACTP–4).

In the case of sheep ACTH hydrolyzate, the active substance was shown in this 5 per cent TCA supernatant fraction, so the hormonal activity of ACTP–4 was estimated at 50 μg. doses, but no activity was found.

Then the precipitate with 5 per cent TCA was also freed from TCA as above and obtained *ca.* 45 mg. of yellowish white hygroscopic powder (ACTP–5). This substance showed almost equal activity to ACTP–1.

Separation of Active Fraction of ACTP–2 with 5 per cent TCA—ACTP–2 was also treated as above and obtained 65 mg. of 5 per cent TCA soluble fraction (ACTP–6), and 30 mg. of precipitate (ACTP–7) from 100 mg. ACTP–2. In this case, activity was also shown in the precipitate only.

From these experiments, it was concluded that the active peptide of HCl hydrolyzate was precipitated with 5 per cent TCA in the case of beef ACTH.

Estimation of the Hormonal Activity of HCl Hydrolyzate—The hormonal activity of ACTP–1～ACTP–7 was estimated by ascorbic acid depletion method. The ascorbic acid content was expressed by mg. per 100 g. adrenal and the ascorbic acid depletion rate was calculated from:

$$R = \frac{\text{ascorbic acid content of left adrenal} - \text{ascorbic acid content of right adrenal}}{\text{ascorbic acid content of left adrenal.}}$$

The results were shown in Table I.

Paper Electrophoresis of the HCl Hydrolyzates—These fractions were studied by paper electrophoresis according to the method of Kunkel *et al.* (*5*).

A 5 cm.×30 cm. strip of the "Toyo" filter paper No. 50 was moistened with

TABLE I

Hormonal Activity of the HCl Hydrolyzate

Fraction	A.A.D.		
	Dose	Rate*	Activity**
	μg.	%	
Original ACTH	500	−19.8	1.0
30 min. Hydrolysis, soluble fraction (ACTP-1)	250	−22.4	1.5
" , insoluble fraction	"	+ 7.0	—
60 min. Hydrolysis (ACTP-2)	"	−15.6	0.5
90 min. " (ACTP-3)	500	+ 5.8	—
ACTP-1, 5% TCA soluble fraction (ACTP-4)	50	+ 3.1	—
" , 5% TCA precipitate (ACTP-5)	"	−15.5	2.0
ACTP-2, 5% TCA soluble fraction (ACTP-6)	"	+ 7.2	—
" , 5% TCA precipitate (ACTP-7)	"	− 7.3	0.7

* A.A.D. rate=ascorbic acid depletion rate.

** The activity of original ACTH is assumed to 1.0.

2 *N* acetic acid and the sample was spotted on the paper 10 cm. from the anode. A potential of 500 to 550 v. was applied for 6 hours in 2 *N* acetic acid as the solvent. After drying, the paper was dipped in a saturated solution of " Amidschwarz 10 B " in a methanol-acetic acid mixture (9:1) for 10 minutes, washed in a methanol-acetic acid solution (9 : 1) several times. The protein fraction which stained blue on the white back ground was cut in 0.5 cm. wide and eluted with 4 ml. of 0.01 *N* NaOH, estimated by Beckmann spectrophotometer at wave length of 575 mμ.

The result was shown in Fig. 1.

From these figures, it is clear that active peptide (ACTP–1 and 2) has a peak I (in the original ACTH), but in ACTP–3, this peak diminished and several small peaks are shown in that position. So the peak I is necessary for the hormonal action of ACTH, but more basic peak II is unnecessary for its hormonal action. (It seemed rather to inhibit the biological action.) The peak I is stained blue by " Amidschwarz 10 B ", while peak II is purple with this dye, so they can be clearly distinguished from each other. Paper chromatography of these peptides was also tried, but clear separation was not attained.

Nitrogen Distribution of HCl Hydrolyzates—Total-N of these fractions were determined by micro Kjehldahl method, and amino-N were determined by the Van Slyke's method and the ratio of both was calculated. The result was shown in Table II. This ratio increased comparatively small in 30 minutes or 60 minutes hydrolysis, but increased largely in 90 minutes hydrolysis indicating the production of small peptides.

FIG. 1. Paper electrophoresis of HCl hydrolyzates.

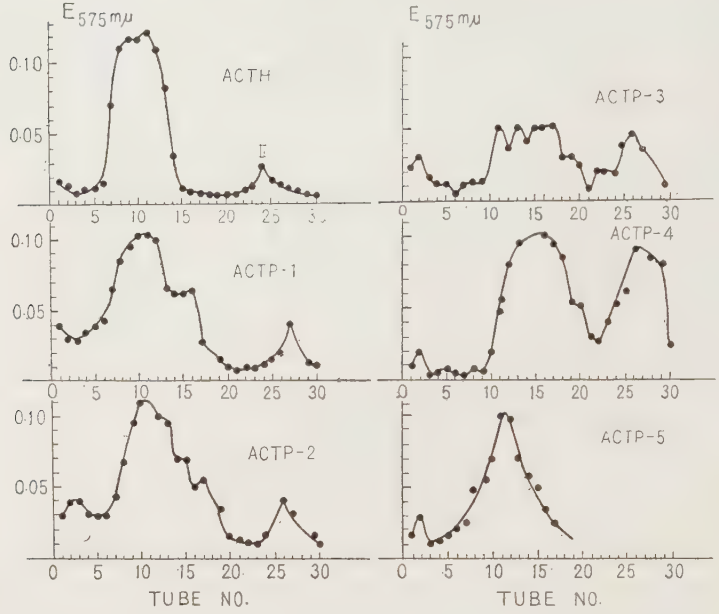

TABLE II

Nitrogen Distribution of the HCl Hydrolyzate

		ACTH	ACTP-1	ACTP-2	ACT-3
Total N	%	13.98	12.85	12.91	12.96
NH_2-N	%	0.78	0.79	0.92	1.74
NH_2-N/Total-N		5.7	6.3	7.2	13.4

DISCUSSION

The beef ACTH retained its hormonal activity when heated in 1 *N* HCl at 100° for 60 minutes. When heated for 30 minutes, the protein was hydrolyzed to 50 per cent, but its hormonal activity increased slightly. At 60 minutes, the protein was hydrolyzed to 70 per cent, but the activity retained to a half. On 90 minutes hydrolysis, it lost all of its activity, amino-N increased from 0.8 to 1.74 per cent.

The active fractions of these hydrolyzates were precipitated with 5 per cent TCA, and on the contrary to Li's data (*1*), the supernatant of 5 per cent TCA solution contained no activity. Paper electrophoretic investigation showed that as the large peak I of the original ACTH decomposed gradually, the biological activity also decreased. When this peak was separated to several smaller fractions, its hormanal activity also diminished. These results indicate that the peak I is the active substance of ACTH and the mare basic peak II is of no influence on the activity, it seemed rather to inhibit the hormonal activity.

As above, we obtained the results different from Li's data, but it is not decided here, whether it is due to the difference in animal species or to the difference in preparative method of original ACTH. But it is clearly demonstrated that the beef ACTH also retains its hormonal activity even when heated in 1 *N* HCl at 100° for 60 minutes.

SUMMARY

Beef ACTH was heated in 1 *N* HCl at 100° for several minutes. To 60 minutes, it retained its activity, but at 90 minutes, its activity was lost. The active peptide of these partial hydrolyzates was precipitated with 5 per cent TCA. The paper electrophoresis and N-distribution were also studied on these samples.

We wish to express our gratitude to Dr. K. Takeda for his kind guidance and encouragements through this investigation, and to Mr. A. Tanaka for his much technical assistance.

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The Journal of Biochemistry, Vol. 42, No. 1, 1955

THE ACTIVE CENTER OF THIAMINE

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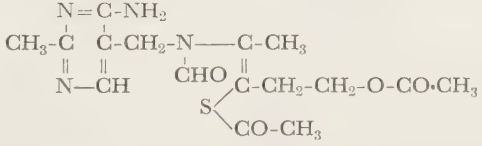
(Received for publication, November 27, 1954)

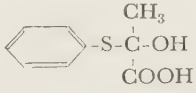
It has been suggested that the lone pair of electrons on the thiazol nitrogen of thiamine pseudobase catalyzes acetoin formation from pyruvate and the diacetylmutase reaction. (*1,2,3,4*) However, Karrer (*5*) has suggested that the first step in pyruvate decarboxylation may involve opening of the thiazol ring to form thiamine thiol with subsequent formation of a thioacetal of pyruvate. Furthermore, Matsukawa *et al.* (*6*) have prepared chemically *O*, *S*-diacetylthiamine, and showed that one of the acetyl groups attached to the thiazol sulfur of the above compound can serve as an acetyl donor. In these circumstances, in this communication some thioacetals and thiol compounds were tested whether they were capable of catalysing acyloin condensation or not.



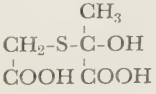
EXPERIMENTAL

Compounds Used—Thiophenol was prepared from benzensulfonyl chloride (*7*).

Thiophenol-pyruvic acid addition compound was synthesized by the method of Baumann (*8*).



Thioglycolic acid-pyruvic acid addition compound was synthesized by the method of Schubert (*9*).



O, S-diacetylthiamine was kindly furnished by Dr. Matsukawa.

Methods—Acetoin was determined by the method of Westerfeld (*10*).

RESULTS

(1) Cysteine was mixed with pyruvate and acetaldehyde in Thunberg tubes, which are evacuated and kept at 37° for one hour. The reaction mixture was distilled in the presence of ferric chloride and sulfuric acid and the amount of acetoin produced was determined colorimetrically. No acetoin formation was observed (Table I).

TABLE I

		(1)	(2)	(3)
0.1 *M* cysteine HCl	*ml.*	1.00	1.00	—
0.1 *M* CH_3·CO·COONa	*ml.*	1.00	1.00	1.00
1.0 *M* CH_3CHO	*ml.*	0.20	0.20	0.20
pH (adjusted by *N* NaOH)		7	8	8
Final volume	*ml.*	3.00	3.00	3.00
Acetoin formed	γ	5.3	5.3	15.0

(2) Thioglycolic acid was incubated with pyruvate and acetaldehyde in the same condition as described above. No acetoin was formed (Table II).

TABLE II

		(1)	(2)	(3)	(4)
0.01 *M* thioglycolic acid	*ml.*	1.00	1.00	1.00	1.00
0.1 *M* CH_3·CO·COONa	*ml.*	1.00	1.00	1.00	1.00
1.0 *M* CH_3CHO	*ml.*	0.20	0.20	0.20	0.20
pH (adjusted by *N* NaOH)		6	7	8	9
Final volume	*ml.*	2.50	2.50	2.50	2.50
Acetoin formed	γ	5.8	8.7	14	13

(3) Thioglycolic acid-pyruvic acid addition compound was incubated with acetaldehyde in the same condition as above. No acetoin was produced (Table III).

TABLE III

		(1)	(2)	(3)	(4)
0.1 *M* CH_3 / CH_2-S-C-OH / COOH COOH	*ml.*	1.00	1.00	1.00	1.00
1.0 *M* CH_3CHO	*ml.*	0.02	0.20	0.20	0.20
pH (adjusted by *N* NaOH)		7	8	9	10
Final volume	*ml.*	2.00	2.00	2.00	2.00
Acetoin formed	γ	15	16	16.5	14

(4) Thiophenol and furfural were dissolved in 50 per cent alcohol. The mixture was boild under reflux condenser for one hour and kept overnight in a refrigerator. Long faint yellow needles appeared were collected and recyrstallized from alcohol. Its melting point was 60° and no depression was observed with a known sample of phenyldisulfide. No furoin (or furil) was obtained (Table IV).

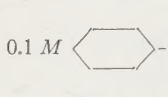
TABLE IV

		(1)	(2)
	*ml.*	1.00	1.0
	*ml.*	0.75	1.5
50% alcohl	*ml.*	15.0	15.0
pH (adjusted by 5*N* NaOH)		9	9
Furoin or furil produced	γ	0	0

(5) Thiophenol-pyruvic acid addition compound was incubated with acetaldehyde in the same condition as in the previous experiment (*1*). No acetoin was produced (Table V).

(6) *O*, *S*-diacetylthiamine was incubated with acetaldehyde in the same condition as above. Large amounts of acetoin were determined, and identified as a nickel dimethylglyoxime. However, the method employed for the determination of acetoin involves an oxidation process of acetoin to diacetyl with a mixture of ferric chloride and sulfuric acid, and

TABLE V

		(1)	(2)	(3)	(4)
0.1 *M* 	*ml.*	1.00	1.00	1.00	1.00
1.0 *M* CH_3CHO	*ml.*	0.20	0.20	0.20	0.20
pH (adjusted by *N* NaOH)		6	7	8	9
Final volume	*ml.*	2.00	2.00	2.00	2.00
Acetoin formed	γ	4	7	13	16

it was found that diacetylthiamine itself gave diacetyl by this oxidation procedure. When diacetylthiamine was oxidized in the presence of acetaldehyde, the amount of diacetyl formed was less than in the absence of acetaldehyde. Furthermore, no acetoin (or diacetyl) was detected, when the incubated mixture described above was distilled without oxidation (Table VI). Therefore, it is concluded that acetoin is not formed from diacetylthiamine and acetaldehyde under the experimental condition.

TABLE VI

		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
0.1 *M* Diacetylthiamine HCl	*ml.*	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1.0 *M* CH_3CHO	*ml.*	0.2	0.2	0.2	0.2	0.2	—	—	—
pH (adjusted by *N* NaOH)		6	7	8	9	7	7	Without incubation, 37°	
Final volume	*ml.*	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Diacetyl formed									
after oxidation	γ	440	470	455	400	—	920	—	840
without oxidation	γ	—	—	—	—	8	—	7	—

DISCUSSION

As previously reported, thiamine was able to catalyze both acetoin formation from pyruvate and furoin condensation from furfural (*11*, *12*) under the condition employed in this paper. However, acyloin con-

densation was never catalyzed by thiols, thioacetals, and *O*, *S*-diacetylthiamine, which might be considered to be an oxidatively decarboxylated produce of thioacetal of pyruvate and thiamine-thiol. Furthermore, it is well known that cyanide is a good catalyst for acyloin condensation. Although cyanide was less active than thiamine, it was found to be able to catalyze the acetoin formation in our model system.

SUMMARY

Cysteine, thioglycolic acid, thioglycolic acid-pyruvic acid addition compound, thiophenol, thiophenol-pyruvic acid addition compound, and *O*, *S*-diacetylthiamine were tested of their ability of acyloin condensation, and it was found that these sulfhydryl compounds could not serve as the catalysts for the aceloin condensation. However, sodium cyanide can catalyze the acetoin formation from pyruvate and acetaldehyde.

This work was done by a fund of the Vitamin B Research Committee.

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